Book

MEETING

SCIENTIFIC ADVISORY BOARD

September 10, 11, 12, 1975

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Book I

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Applications to be considered

A. Renewals

B. New

   Cancer

   Cardiovascular

   Epidemiology

   Pharmacology

Book II

Pulmonary Pulmonary

   Miscellaneous

   Deferrals

Book III

Georgetown University

   Hamosh

   Herscowitz

   Vidic

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AGENDA

SCIENTIFIC ADVISORY BOARD
September 10, 11, 12, 1975

1. Interim Actions for Ratification

   a. Administrative Actions
   b. Renewal Applications

2. Applications to be Considered for Grants

   Book I - Renewals

   Cancer

   Cardiovascular

   Epidemiology

   Pharmacology

   Book II - Pulmonary

   Miscellaneous

   Deferrals

   Book III - Georgetown University

3. Other business

4. Future meeting dates

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

CONFIDENTIAL REPORT

SCIENTIFIC ADVISORY BOARD MEETING

SANTA BARBARA, CALIFORNIA

MARCH 26-27-28, 1975

Attendance

Dr. Sheldon C. Sommers, Chairman	
Dr. Richard J. Bing (3/26-27)	
Dr. Joseph D. Feldman	
Dr. William U. Gardner	Scientific Director, CTR
Dr. Robert J. Huebner	
Dr. Leon O. Jacobson	
Dr. Averill A. Liebow	
Dr. Henry T. Lynch	
Dr. John P. Wyatt	
Dr. Helmut R. R. Wakeham	Chairman, ITC
Joseph L. Goldstein, M.D. (3/26)	University of Texas, Southwestern Med. Sch.
Jay A. Levy, M.D. (3/28)	University of California, San Francisco
George J. Popjak, M.D. (3/26)	University of California, Los Angeles
J. S. M. Sarma, Ph.D. (3/26)	Huntington Memorial Hospital
Mr. H. H. Ramm	President, CTR
Mr. W. T. Hoyt	Executive Vice President, CTR
Dr. Robert C. Hockett	Research Director, CTR
Dr. John H. Kreisher	Associate Research Director, CTR
Dr. David Stone	Associate Research Director, CTR
Dr. Vincent F. Lisanti	Research Associate, CTR
Mr. Leonard S. Zahn	Leonard Zahn and Associates, Inc.

1. The report of the meeting of the Scientific Advisory Board, held on October 30-31, November 1, 1974 was approved.

2. The report or the meeting of the Executive Committee of the SAB, held on December 13, 1974, was approved.

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3. The following administrative actions were approved as attached:

Authorizations

Subsequent to the October 1974 Advisory Board meeting, of the new applications recommended for approval, the Scientific Director determined that the following should be funded in the respective amounts indicated:

983 Leo G. Abood, Ph.D. $16,771.00 for ten months or at a rate of $20,125.00 per year.

1003 Edward L. Klaiber, M.D. $20,332.00

Subsequent to the October 1974 SAB meeting, of the renewal applications recommended for approval, the Scientific Director determined that the following should be funded in the amount indicated:

24PS4 Paul S. Larson, Ph.D. $66,753.00

Subsequent to the October 1974 SAB meeting the Scientific Director determined that the following proposal for contract renewal should not be funded:

2-G Microbiological Associates Inc. $181,560.00 (Animal carcinogenic models.)

However, in order to permit completion of some phases of the contract not completed under Contract 2-F, the Scientific Director determined that the following supplement should be funded for six months in the amount indicated:

14-AS Microbiological Associates Inc. $55,000.00

Subsequent to the October 1974 SAB meeting, of the new applications that were deferred, the Scientific Director, with the approval of the Executive Committee of the SAB, determined that the following should be funded in the amount indicated:

787-BS Gary D. Friedman, M.D. $24,860.00 for three months.

Actions

Contract

9-DS University of San Francisco. At the Executive Committee meeting of the SAB held on December 13, 1974, the supplement authorized was incorrectly noted in the minutes as $20,117.00. The final amount authorized is $20,101.02.

Grants

572-CS Jay A. Levy, M.D. $10,000.00 On page 10 of the minutes of October 30-31, November 1, 1974, the above authorization should have indicated grant #572-CS instead of #1011.

766-A Richard A. Lerner, M.D. An unexpended balance of $3.02 was returned and deposited.

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776-AM Elliot S. Vesell, M.D. Authorization was given to rebudget $3,210.00 from Volunteer Fees to Salaries.

814R2 Una S. Ryan, Ph.D. Permission was granted to carry forward an unspent balance of $6,310.00 to grant #814-A toward the purchase of a new LKB Ultratome III.

906S Robert E. Brooks, Ph.D. $621.45 This supplement was approved to cover a deficit incurred in the first year of a proposed three-year project which was terminated at the end of the first year.

923 Guenther Boden, M.D. Permission was granted to carry forward unexpended funds of $12,053.09 on hand at December 31, 1974 to grant #923R1, the second year of the project.

929 Edward Leete, Ph.D. Permission was granted to carry forward an unspent balance of $6,978.84 beyond the expiration date of December 31, 1974 to December 31, 1975, with the proviso that a final financial report be received as soon as possible.

1013 Malcolm C. Pike, Ph.D. The final award was approved at $68,581.00 instead of $63,899.00, reflecting a 12% fringe benefit figure on salaries and wages which had been overlooked.

4. The following renewal applications were recommended for approval subject to the determination by the Scientific Director as to priority and the amount to be funded:

455-BR2 Charles L. Rose, Ph.D. (formerly Bell) $64,000.00

467-CR1 Thomas C. Westfall, Ph.D. $30,597.00

519-CR1 Louis A. Soloff, M.D. $80,329.00

870R2 H. J. Eysenck, Ph.D. £9,315.

884MR1 Albert Castro, Ph.D. $40,120.00

901MR1 George Weinbaum, Ph.D. $40,538.00

918R1 H. Fred Downey, Ph.D. $15,784.00

922MR1 Joseph C. Arcos, D.Sc. $67,279.00

933R1 Charles R. Shaw, M.D. $53,353.00

935MR1 William H. Fishman, Ph.D. $51,368.00

939R1 R. G. Mason, M.D. $28,285.00

941R1 Henry T. Lynch, M.D. Part I. $38,908.00
Part II. $36,515.00

949R1 B. V. Rama Sastry, Ph.D. $14,107.00

951R1 Hans Meier, D.V.M. $27,010.00

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961R1 Dov Michaeli, Ph.D. $84,966.00

966R1 Nathan H. Sloane, Ph.D. $11,500.00

973R1 Ronald E. Rasmussen, Ph.D. $51,446.00

974R1 Stephen F. Vatner, M.D. $27,106.00

985R1 Gary Friedman, M.D. $90,340.00

5. Of the new applications deferred at the meeting of the SAB on October 30-31, November 1, 1974, the following were recommended for approval subject to the determination by the Scientific Director as to priority and the amount to be funded:

787-C Gary Friedman, M.D. $111,900.00

1010M Carl G. Becker, M.D. $29,908.00

1011 Jay A. Levy, M.D. $60,245.00

6. Of the applications deferred at the meeting of the SAB on October 30-31, November 1, 1974, the following was rated for disapproval:

966 John A. Terzakis, M.D. $15,180.00

7. The following new applications were recommended for approval subject to the determination by the Scientific Director as to priority and the amount to be funded:

725-D Jack Chalon, M.D. $25,254.00

764-D Charles G. Cochrane, M.D. $25,818.00

766-B Richard A. Lerner, M.D. $79,178.00

862-A James Travis, Ph.D. $45,081.00

864-C Theodore A. Slotkin, Ph.D. $18,359.00

869-A Ronald P. Rubin, Ph.D. $18,975.00

892-A Lars Friberg, M.D. $43,923.00

954-A Allen B. Cohen, M.D., Ph.D. $65,762.00

1012 J. Andrew Mitchell, Ph.D. $6,887.00

1014 G. J. Gleich, M.D. $8,345.00

1015 Alphonse A. Ingenito, Ph.D. $29,910.00

1020 Marc D. Thames, M.D. $12,500.00

1022 John L. Egle, Ph.D. $29,813.00

1023 Patricia I. Bader, M.D. $52,385.00

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1027 William F. Benedict, M.D. $43,513.00

1028 George Wolf, Ph.D. $23,168.00

8. The following new applications were rated for disapproval:

547-E Joseph J. Guarneri, Ph.D. $45,713.00

976M Stephen M. Ayres, M.D. $41,611.00

1016 William Ho, M.A. $55,481.00

1017 Gordon W. Gribble, Ph.D. $21,090.00

1018 William E. Connor, M.D. $37,605.00

1019 Simon H. Wender, Ph.D. $23,449.00

1024 Ronald D. Fletcher, Ph.D. $19,847.00

9. The following new applications were deferred:

310-A Richard J. Bing, M.D. $46,971.00 Staff is to arrange for peer review with final decision to be made by the Executive Committee of the SAB or pro rated for decision at the September SAB meeting.

758-C Hans Meier, D.V.M. $24,973.00 Staff is to arrange for peer review with final decision to be made by the Executive Committee of the SAB or pro rated for decision at the September SAB meeting.

1021 Giles D. Filley, M.D. $43,522.00 For consideration at September meeting.

1025 Charles Mittman, M.D. $7,877.00 For consideration at September meeting.

1026 Ze'ev Trainin, D.V.M. $31,175.00 For consideration at September meeting.

10. The following proposals for contract renewal were recommended for approval subject to the determination by the Scientific Director as to priority and the amount to be funded:

14-B Microbiological Associates Inc. $522,794.00

15-B Union Carbide Corporation $368,300.00

16-B Scripps Clinic and Research Foundation. (Richard A. Lerner, M.D. $65,872

20-A Microbiological Associates Inc. $25,000.00 (Reference cigarette storage.)

22-A Microbiological Associates Inc. $900,000.00

28 Microbiological Associates Inc. $40,685.00 (Kouri collaboration with Jay A. Levy, M.D. [grant 1011].)

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11. The next meeting of the Scientific Advisory Board was scheduled for September 10-11-12, 1975.

12. Dr. S. C. Sommers was reelected Chairman of the Scientific Advisory Board for a term of one year.

13. Following a discussion of the advisability of having terms of office for Scientific Advisory Board members, the Scientific Director was asked to collaborate with the Chairman of the Advisory Board in formulating a procedure covering members' tenure on the Board.

14. Subsequent to the meeting, of the renewal applications recommended for approval and referred to in paragraph 4, the Scientific Director determined that the following should be funded in the respective amounts indicated:

455-BR2	Charles L. Rose, Ph.D. $64,000.00
467-CR1	Thomas C. Westfall, Ph.D. $30,597.00 Terminal.
519-CR1	Louis A. Soloff, M.D. $69,167.00 Terminal.
870R2	H. J. Eysenck, Ph.D. $22,000.00 Terminal.
884MR1	Albert Castro, Ph.D. $30,090.00 4/1-12/31/75
901MR1	George Weinbaum, Ph.D. $40,538.00
918R1	H. Fred Downey, Ph.D. $15,784.00
922MR1	Joseph C. Arcos, D.Sc. $56,000.00 No commitment.
933R1	Charles R. Shaw, M.D. $28,000.00 4/1-12/31/75
935MR1	William H. Fishman, Ph.D. $51,368.00
939R1	R. G. Mason, M.D. $28,285.00
941R1	Henry T. Lynch, M.D. $68,000.00 Includes Parts I and II.
949R1	B. V. Rama Sastry, Ph.D. $14,107.00 Terminal.
951R1	Hans Meier, D.V.M. $27,010.00
961R1	Dov Michaeli, Ph.D. $74,000.00
966R1	Nathan H. Sloane, Ph.D. $11,500.00
973R1	Ronald E. Rasmussen, Ph.D. $51,446.00 No commitment.
974R1	Stephen Vatner, M.D. $27,106.00
985R1	Gary Friedman, M.D. $90,340.00 Terminal.

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15. Subsequent to the meeting, of the new applications recommended for approval and referred to in paragraph 7, the Scientific Director determined that the following should be funded in the respective amounts indicated:

725-D	Jack Chalon, M.D.	$25,254.00
764-D	Charles G. Cochrane, M.D.	$25,818.00
766-B	Richard A. Lerner, M.D.	$55,000.00
862-A	James Travis, Ph.D.	$45,081.00
892-A	Lars Friberg, M.D.	$43,923.00
954-A	Allen B. Cohen, M.D., Ph.D.	$58,326.00
1012	J. Andrew Mitchell, Ph.D.	$6,887.00
1014	G. J. Gleich, M.D.	$8,345.00
1015	Alphonse A. Ingenito, Ph.D.	$29,910.00
1020	Marc D. Thames, M.D.	$12,500.00
1027	William F. Benedict, M.D.	$43,513.00
1028	George Wolf, Ph.D.	$23,168.00

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16. Subsequent to the meeting, of the renewal contract proposals recommended for approval and referred to in paragraph 10, the Scientific Director determined that the following should be funded in the respective amounts indicated:

14-B	Microbiological Associates Inc.	$480,000.00
15-B	Union Carbide Corporation	$260,000.00
20-A	Microbiological Associates Inc.	$25,000.00 (Reference c'ette. storage.)
16-B	Scripps Clinic & Research Foundation (Richard A. Lerner, M.D.)	$65,872.00
22-A	Microbiological Associates Inc.	$900,000.00
28	Microbiological Associates Inc.	$40,685.00 (Kouri collaboration with Jay A. Levy, M.D., [grant 1011].)

17. Subsequent to the meeting, of the deferred applications referred to in paragraph 5, the Scientific Director determined that the following should be funded in the respective amounts indicated:

787-C	Gary Friedman, M.D.	$49,720.00	5/1-10/31/75
1011	Jay A. Levy, M.D.	$60,245.00	

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18. Subsequent to the meeting, of the deferred applications referred to in paragraph 5, the Scientific Director determined that the following should not be funded:

1010M	Carl G. Becker, M.D.	$29,808.00

19. Subsequent to the meeting, of the new applications recommended for approval and referred to in paragraph 7, the Scientific Director determined that the following should not be funded:

864-C	Theodore A. Slotkin, Ph.D.	$18,359.00
869-A	Ronald P. Rubin, Ph.D.	$18,975.00
1022	John L. Egle, Ph.D.	$29,813.00
1023	Patricia I. Bader, M.D.	$52,385.00

20. Subsequent to the meeting, of the new applications that were deferred to in paragraph 9, the Scientific Director determined that the following should be prorated until the September SAB meeting in the respective amounts indicated:

310-A	Richard J. Bing, M.D.	$11,323.25	7/1-9/30/75
758-C	Hans Meier, D.V.M.	$6,100.25	7/1-9/30/75

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ADMINISTRATIVE ACTIONS

864-A	Theodore T. Slotkin, Ph.D. An unexpended balance of $10.02 was returned and deposited.
24R18	Paul S. Larson, Ph.D. Permission was granted to carry forward an unspent balance beyond the expiration date of June 30, 1975 for the purpose of completing work on Supplement III to "Tobacco."
884M	Albert Castro, Ph.D. Permission was granted to carry forward an unspent balance of approximately $1,719.62 beyond the expiration date of March 31, 1975 to grant 884MR1.
Contracts 9-C 9-DS2	University of San Francisco. Permission was granted to carry forward an unspent balance on hand at May 31, 1975 to Contract 9-DS2 with the proviso that a final financial report be received as soon as possible after the expiration date of October 11, 1975.
547-CR2	Joseph J. Guarneri, Ph.D. An extension without additional funds was authorized from April 1 to June 30, 1975.
869R1	Ronald P. Rubin, Ph.D. An extension without additional funds was authorized from July 1 to December 31, 1975.
1003	Edward L. Klaiber, M.D. An extension without additional funds was authorized from March 1, 1976 to May 31, 1976.
642-D	Leonide Goldstein, D.Sc. Permission was granted to rebudget approximately $3,069.00 for Permanent Equipment with Rutgers Medical School agreeing to accept an overhead of 5% rather than 15% allowed by The Council. A revised budget was requested and reference was also made to the unspent balance of $997.02 remaining as of December 31, 1973 for which no report has been received.
814-A	Una S. Ryan, Ph.D. Permission was granted to rebudget $816.42 from other categories to Travel.
862-A	James A. Travis, Ph.D. Permission was granted to rebudget $3,000.00 with the proviso that no additional funds are requested. A revised budget was requested.
923R1	Guenther Boden, M.D. Permission was granted to rebudget funds from other categories to Travel and a revised budget was requested.
939	R. G. Mason, M.D., Ph.D. Permission was granted to alter the budget as outlined and a request of expenditures was requested as soon after the termination date (June 30, 1975) as possible.

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1011 Jay A. Levy, M.D. Permission was granted to rebudget $444.62 from other categories to Travel.

1027 William F. Benedict, M.D. Permission was granted to rebudget $2,894.00 from Personnel to Permanent Equipment.

599-CR1S Domingo M. Aviado, M.D. $19,440.00 This supplement was approved to extend the grant for the period July 1 through December 31, 1975.

878R2S Paul Hamosh, M.D. $2,700.00 Approved.

936R1S Branislav Vidic, D.S. $4,800.00 Approved.

954S Allen B. Cohen, M.D. $5,326.00 This supplement was approved to extend the grant for the period May 1 through June 30, 1975.

Contracts

9-DS2 University of San Francisco. $40,416.00 This supplement was approved to extend the grant for the period May 12 to November 10, 1975.

18-A University of Kentucky. $17,000.00 Approved for production of 1A3 reference cigarettes needed in conjunction with Council contracts.

24S Meloy Laboratories, Inc. $9,800.00 Approved for production of smoke condensate needed in conjuncation with Council contracts.

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RENEWAL APPLICATIONS

No.	Name and Title	Request	Committee
983R1	Leo G. Abood, Ph.D. "Behavioral Effects of Nicotine and Piperiduie Using a Computer-Controlled Program"	$ 21.275.00	Gardner, Meier, Sommers
884MR2	Albert Castro, Ph.D. "Nicotine in Blood: Detection by Radioimmunoassay"	$ 46,383.00	Gardner, Jacobson, Sommers
937R2	Carroll Cross, M.D. "Cigarette Smoke Effects on Certain Aspects of Rat Lung Metabolism"	$ 40,000.00(?)	Jacobson, Bing, Sommers, Wyatt
986R1	J. W. Gorrod, D.C.C. "The Metabolism of 'Pyridines' in Relation to the Induction of Neoplastic Disease"	$ 12,640.00	Gardner, Huebner, Jacobson
914R2	Michael E. Lamm, M.D. "Immune Mechanisms of Mucous Membranes"	$ 36,723.00	Bing, Feldman, Huebner, Wyatt
953MR1	Ilari Rantasalo, M.D. "The Finnish Twin Registry"	$ 85,202.00	Gardner, Jacobson, Lynch
928R2	Daniel B. Rifkin, Ph.D.	$ 42,846.00	Gardner, Jacobson, Sommers, Wyatt
909R2	John A. Rosecrans, Ph.D. "State Dependent Properties of Nicotine Related Compounds"	$ 20,471.00	Gardner, Meier, Sommers
814AR1	Una S. Ryan, Ph.D. "Endocrine Functions of the Lungs"	$ 44,993.00	Bing, Feldman, Sommers
936R2	Branislav Vidic, S.D. "The Effect of Cigarette Smoke on Lung Metabolism"	$ 56,736.00	Gardner, Sommers, Meier, Wyatt

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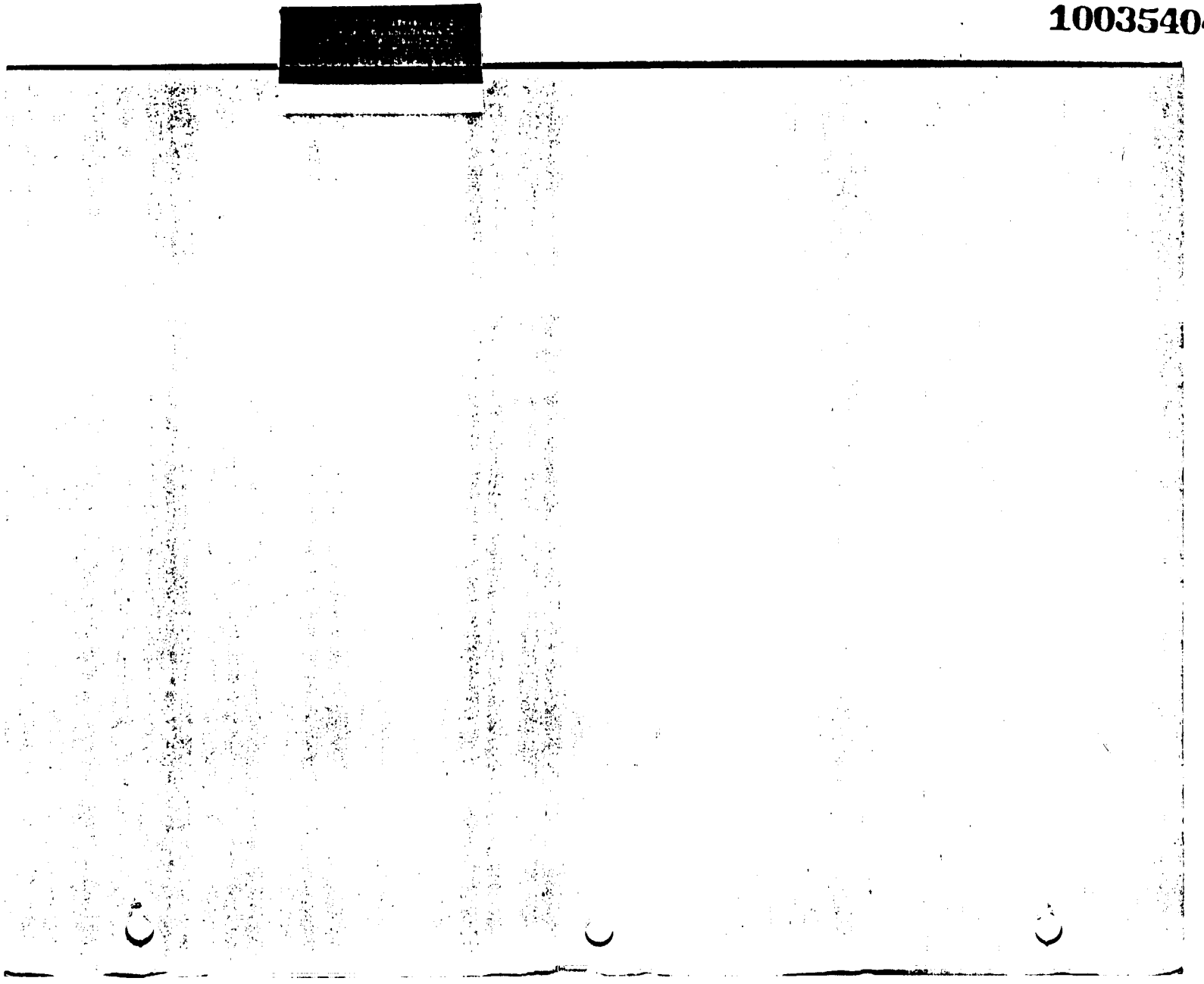

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#1013R1 - PIKE

1003540500

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

May 1, 1975

Grant application No. 1013R1

CANCER

To: The committee comprising Drs. Gardner, Heubner, Jacobson and Lynch

Subject: M. C. Pike, Ph.D., University of Southern California, Los Angeles
Renewal application No. 1013R1
"Study of relationship between susceptibility to certain cancers and aryl hydrocarbon hydroxylase (AHH) activity"

History

Originally this study, involving a collaborative effort between the University of Southern California (Pike) and MAI (Kouri), was supported by Contract No. 23 at a level of $20,000 for the period 7/1/74 - 10/31/74. Application for further support, in the form of grant application No. 1023, was approved by the SAB in October 1974 in the amount of $68,581.

Request

Application No. 1013R1 requests $69,900.

Documents Submitted (attached)

1. Application dated 4/21/75 (4 pages)

2. CV of Dr. Rasheed (8 pages)

3. Progress report for the period November 1, 1974 - April 30, 1975 (14 pages including tables and figures).

Comment

This request will be handled administratively and withheld from the bulky SAB agenda for the September 1975 meeting, unless you object.

D.S.

DS/lp
Encl.

1003540501

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UNIVERSITY OF SOUTHERN CALIFORNIA
UNIVERSITY PARK
LOS ANGELES 90007

VICE PRESIDENT
ACADEMIC ADMINISTRATION AND RESEARCH

In reply refer to:181045-M/C

April 21, 1975

The Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

SUBJECT: Proposal - "Study of relationship between susceptibility to certain cancers & aryl hydrocarbon hydroxylase (AHH) activity"

Principal Investigator: Malcolm C. Pike, Ph.D.
Amount Requested: $69,900
Period: 11/1/75 through 10/31/76
Number of Copies: Orininal

Gentlemen:

On behalf of the University of Southern California, I am pleased to submit the attached proposal for your review and consideration. We shall look forward to notification of the award for funding for this most important research as described in the attached proposal.

Please address all official correspondence to the attention of Clark A. McCartney, Director, Department of Contracts and Grants, at the above address.

Very truly yours,

Zohrab A. Kaprielian
Vice President
Academic Administration and Research

ZAK:CAM:ss

Enclosures:

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1013 R1

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
APR 25 1975

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐ Second Renewal ☒ Date: 4/21/75

1. Principal Investigator (give title and degrees): M.C. Pike, Ph.D.
Professor, Community Medicine and Pediatrics

2. Institution & address: University of Southern California
School of Medicine
2025 Zonal Avenue
Los Angeles, California 90033

3. Department(s) where research will be done or collaboration provided: Department of Pathology

4. Short title of study: Study of relationship between susceptibility to certain cancers and aryl hydrocarbon hydroxylase (AHH) activity

5. Proposed renewal date: November 1, 1975

6. How results to date have changed earlier specific research aims:

No change in basic epidemiological research aims.

7. How results to date have changed earlier working hypothesis:

The failure of both our collaborator Dr. Kouri at Microbiological Associates and ourselves to produce a reproducible AHH assay using human peripheral blood lymphocytes has caused us to change (temporarily?) from these cells to cultured cell lines of different types for assay purposes (see attached Progress Report).

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7. Any additional facilities now required? Describe briefly.

None

8. Any changes in personnel? Append biographical sketches of new key professional personnel.

The key personnel in this research are:

M.C. Pike, Ph.D.
J.C. Brown, M.D.
R.J. Gordon, Ph.D.
S. Rasheed, Ph.D. (C.V. appended)

10. Append outline of experimental protocol for ensuing year. See attached

11. List publications or papers in press resulting from this or closely related work. (append reprint or manuscripts not previously sent).

None

12. Summary progress report (append in standard form as seperate document, unless recently submitted).

See attached

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Professional		
M.C. Pike, Ph.D.	20	---
J.C. Brown, M.D.	20	---
R.J. Gordon, Ph.D.	10	---
S. Rasheed, Ph.D.	5	---
Technical		
S. Brown, Tech I	100	REDACTED
R. Hanisch, R.N., Nurse Epidemiologist	100	
H. Lewis		
a. Tech II 7/75-6/76 (R /yr)		
b. Promotion Tech III 7/76-10/76 (R /yr)	100	
Subtotal		REDACTED
Projected 10% salary increase for period July 1 - October 31, 1976 (base $11,784)		1,178
		36,230
12% of salaries and wages ($36,230)		4,348
Sub-Total for A		$40,578
B. Consumable supplies (by major categories)		
Supplies for shipments, boxes, wrappings, tubes and needles		858
Glassware, chemicals, media, automatic pipettes and miscellaneous laboratory supplies		14,520
6% tax (base $15,378)		923
Sub-Total for B		16,301
C. Other expenses (itemize)		
Specimen shipments to Microbiological Associates - $17.33/shipment×3 shipments×52 weeks		2,704
Travel: An estimated 10,000 miles @ 12¢/mile		1,200
Sub-Total for C		3,904
Running Total of A + B + C		60,783
D. Permanent equipment (itemize)		
None		
Sub-Total for D		—
E. Indirect costs (15% of A+B+C)		9,117
Total request		69,900

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Comprehensive Field and Laboratory Research Program on the Etiology and Epidemiology of Human Cancer	N.C.I. Contract PH43-NCI-68-1030	2628354	7/1/74 - 6/30/75 Renewal Pending

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Cancer Center Epidemiology and Biostatistics Unit	N.C.I. Grant POICA 10754-01	565913 annually	1/6/75 - 12/31/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name M.C. Pike, Ph.D.

Signature [signature] Date 4/21/75

Telephone 213 (Area Code) 223-1379 (Number) (Extension)

Checks payable to University of Southern California School of Medicine

Mailing address for checks:

2025 Zonal Avenue

Los Angeles, California 90033

Attention: Gary King

Responsible officer of institution

Typed Name Z.A. Kaprielian

Title Vice President, Academic Administration & Research

Signature [signature] Date APR 18 1975

Telephone 213 (Area Code) 226-2033 (Number) (Extension)

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June 6, 1974

CURRICULUM VITAE

A. Personal Information:

Name in Full	Suraiya Rasheed, Ph.D.
Business Address	Department of Pathology USC School of Medicine 1840 North Soto Street Los Angeles, California 90032
Business Phone	226-2135
Home Address	
Home Phone	REDACTED
Date of Birth	
Place of Birth	
Citizenship	
Marital Status	
Spouse's First Name	REDACTED
Number of Children	
Social Security Number	

B. Education:

University	Osmania University, Hyderabad, India, B.Sc., 1953 Osmania University, Hyderabad, India, M.Sc., 1955 Osmania University, Hyderabad, India, Ph.D., 1958 London University (School of Hygine and Tropical Medicine), London, Ph.D., 1964 University of California, Irvine, Certificate in continuing Medical Education on course in Feline Leukemia-Sarcoma and Myeloproliferative disease, 1971.
Honors and Awards	Awarded Post-Doctoral Fulbright Scholarship, 1961, Declined Awarded Post-Doctoral Commonwealth Scholarship to complete Ph.D. at London University, 1961 Invited Speaker and Delegate from England for the First International Congress of Parasitology in Rome, 1964.

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C. Professional Background:

Academic Appointments

Lecturer of Microbiology and Parasitology, Women's College, Osmania University, Hyderabad, India, 1956-57 (part time)
Senior lecturer of Microbiology and Parasitology, Warangal College, Osmania University, Hyderabad, India, 1957-58
Senior lecturer and department head, PECHS College, Karachi, West Pakistan, 1958-59
Microbiologist/Helminthologist, Zoological Survey Department, Government of West Pakistan, 1959-61
Wellcome Laboratories, in vitro culture of tissue, viruses and parasites, 1961-64, (part time)
Research Associate in Department of Cancer Research, Mount Vernon Hospital, Northwood, Middlesex, England, 1964-70
Instructor in Pathology, University of Southern California School of Medicine, 1971-72
Assistant Professor of Pathology, University of Southern California School of Medicine, 1972-present

Specific teaching responsibilities

Lectures in Microbiology and Parasitology for graduate and undergraduate students, 1956-57 (part time)
Review of Parasitology course for M.S. students 1959-61
Lectures in Viral Oncology for graduate students, 1972-present

Specific administrative responsibilities

Department Head (Science), PECHS College, Karachi, West Pakistan, 1958-59
Section Head, Zoological Survey Department, Government of West Pakistan, 1959-61
Section Head, Viral Oncology Program, Department of Pathology, University of Southern California School of Medicine, 1971-present

D. Society Memberships:

National

REDACTED

REDACTED

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E. Consultantships:

Local

Dr. Goerge Friou's tissue culture/virology laboratory at the University of Southern California School of Medicine

Post doctoral training in tissue culture/virology of Dr. Ira Roy in Dr. Peters' Department of Pathology at John Wesley Hospital, Los Angeles

F. Research Activities:

Complete Bibliography
Appended

Major Areas of Research Interest

RNA tumor virology and tissue culture, cancer etiology.

Research in Progress

Isolation of viruses from various mammalian tissues, including cat, rat, mouse and humans. Analysis and characterization of tumor viruses.

Viral-chemical cocarcinogenesis in vitro

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2. Rasheed, S.
A Revision of the Genus Philometra Costa, 1945.
Journal of Helminthology 37: 89-130, 1963.

3. Rasheed, S.
On Hepatinema Karachiensis, gen. et. sp. nov. Thelaziidae Railliet 1916 from a Marine Fish of Pakistan, with a Note on Genus Filochona (Saidov, 1954 Yamaguti, 1961)
Journal of Helminthology 38: 63-76, 1964.

4. Powell, A. K., Rasheed, S. and Wright, J.
Carcinogenic Action of Propiolactone on Cells Cultivated In Vitro.
British Empire Campaign for Research 42nd Report Part II, 207-209, 1964.

5. Rasheed, S.
On Remarkable New Nematode, Lappetascaris Lutjani, gen, et. sp. nov. Anisakidae; Ascaridoidea from Marine Fishes of Karachi and An Account of Thynnascaris Inquiries (Linton, 1901) N. Comb. and Goezia Intermedia N. Sp.
Journal of Helminthology 39: 313-342, 1965.

6. Rasheed, S.
Additional Notes on the Family Philometridae Baylis and Daubney, 1926 (Dracunculoidea).
Journal of Helminthology 39: 349-362, 1965.

7. Rasheed, S.
Some Parasitic Nematodes from the Cameroons (W. Africa).
Journal of Helminthology 39: 67-100, 1965.

8. Rasheed, S.
A Preliminary Review of the Genus Rhabdochona Railliet, 1916 with Description of a New and Related Genus.
Acta Parasitologica Polonica Warsaw, 13, 42: 407-424, 1965.

9. Rasheed, S.
On Cyrnea (Procyrnea) Chabaudi from a Fish Parastronateus sp. in Pakistan; the First Record of a Habronematid Parasite in a Piscine Host.
Journal of Helminthology 39: 343-348, 1965.

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10. Powell, A. K., Rasheed, S. and Wright, J.
Effects of Carcinogens on Cells Cultured In Vitro.
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11. Rasheed, S.
Observations on the Spiruroid Nematodes of Fish with a Revision of the Genus Metabronema Yorke and Maplestone, 1926.
Zeitschrift Zool Syst, Evolutions-Forschung 3: 359-387, 1966.

12. Rasheed, S.
On Some Interesting Nematode Parasites of Fish from Pakistan.
Parasitology 56: 151-160, 1966.

13. Rasheed, S.
The Nematodes of the Genus Cacullanus Mueller, 1777 from the Marine Fish of Karachi Coast.
Anales de la Escuela Nacional de Ciencia Biologica, Mexico 15: 23-59, 1966.

14. Rasheed, S.
Effects of Maleic Hydrazide on the Rat Lung Grown In Vitro.
British Empire Cancer Campaign for Research, 44th Report, Part II, 121, 1966.

15. Rasheed, S.
Effects of Urethane on Cells Cultivated In Vitro.
British Empire Cancer Campaign for Research, 44th Report, Part II, 120, 1966.

16. Rasheed, S.
Effects of S 37 Plasma on Cells Cultivated In Vitro I.
British Empire Cancer Campaign for Research 46th Report, 129-130, 1967.

17. Rasheed, S.
Effects of S 37 Plasma on Cells Cultivated In Vitro II.
British Empire Cancer Campaign for Research, 47th Report, 162, 1969.

18. Rasheed, S.
Morphological Transformation of Mouse and Rat Embryo Cells In Vitro by an Agent from S 37 Ascites Tumour.
British Journal of Cancer 25: 142-148, 1971.

19. McAllister, R. M., Nicolson, M., Gardner, M. B., Rongey, R. W., Rasheed, S., Sarma, P. S., Huebner, R. J., Hatanaka, M., Oroszlan, S., Gilden, R. V., Kabigting, A. and Vernon, L.
C-Type Virus Released from Cultured Human Rhabdomyosarcoma Cells.
Nature New Biology 235: 3-6, 1972.

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20. McAllister, R. M., Nicolson, M., Gardner, M. B., Rasheed, S., Rongey, R. W., Hardy, W. D. and Gilden, R. V.
RD-114 Virus Compared with Feline and Murine Type C Viruses Revealed from RD Cells.
Nature 242: 75-78, 1973.

21. Rasheed, S., McAllister, R. M., Henderson, B. E., and Gardner, M. B.
In Vitro Host Range and Serological Studies on RD-114 Virus
Journal of the National Cancer Institute 51: 1383-1385, 1973

22. Gardner, M. B., Johnson, E. Y., Rasheed, S. and McAllister, R. M.
Intracerebral Transplantation of Human Rhabdomyosarcoma Cells into Fetal and Newborn Kittens.
International Journal of Cancer 12: 563-567, 1973.

23. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. and Gardner, M. B.
Characterization of a Newly Derived Human Sarcoma Cell Line (HT-1080).
Cancer 33: 1027-1033, 1974.

24. Gardner, M. B., Rasheed, S., Rongey, R. W., Charman, H. P., and Alena, B.
Natural Expression of Feline Type C Virus Genomes, Prevalence of Detectable FeLV and RD-114 gs Antigen, Type C Particles and Infectious Virus in Postnatal and Fetal Cats.
International Journal of Cancer 14: 1974.

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Suraiya Rasheed, Ph.D.

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ABSTRACTS

1. Rasheed, S., Charman, H. P., Bruszewski, J., Roy-Burman, P. and Rongey, R. W.
   Cultures of Two Different Rat Strains.
   Amer. Soc. Micro. p 263
   Paper presented at ASM Meeting in Chicago, May, 1974

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Suraiya Rasheed, Ph.D.

PRESENTATIONS

1. 1971, Department of Pathology Seminars:
   Trichinella Infection in Man and Animals
   Isolation of a Transforming Agent from the Plasma of S37 ascites tumor of BALB/c Mouse

2. 1972 June, PACTVIGAR Meeting
   Transformation of Fibroblast from Kaposi Sarcoma by RD-114 Virus

3. 1973 ASM - Florida - A short discussion on the effects of RD-114 Virus on Mammalian Cell Cultures In Vitro

4. 1974 April, PACTVIGR Meeting
   Attempts to Isolate Type C Virus from Human Placenta

5. 1974 ASM - Chicago
   Spontaneous Release of Type C Virus in Primary and Secondary Embryo Cultures of Two Different Rat Strains

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STUDY OF RELATIONSHIP BETWEEN SUSCEPTIBILITY TO VARIOUS CANCERS AND ARYL HYDROCARBON HYDORXYLASE (AHH) INDUCIBILITY

PROGRESS REPORT

April 21, 1975

Period covered: November 1, 1974 - April 30, 1975

Summary:

Our essential aim is to test the published results of Shaw and the Kellerman's that an individuals AHH inducibility is strongly related to his lung cancer risk.

The core problem of this study still remains to be solved as of this date, viz. the establishment of a repeatable test. As we stated in our previous report the chemistry part of the test has been satisfactorily established and we have made further improvements in it recently. But the variability of AHH inducibility in peripheral blood lymphocytes between split-samples of bloods run in parallel is much too great for comfort, and the day-to-day variation is considerably worse: this is true whether the analysis is done in Dr. Kouri's laboratory or in our own laboratory.

Dr. Kouri continues to work on the AHH assay of peripheral blood lymphocytes, but, because of these problems, we have shifted recently from these cells to cultured cell lines. This change has given us a continuing source of "identical" cells to work with and, although the AHH activity is no greater than stimulated lymphocytes, we have managed to produce much less variable results and to identify the main sources of variation in results.

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We should be able during the next few months to establish a straightforward, easily duplicated and repeatable test for AHH inducibility in fibroblast cell lines. Although this test will clearly be considerably more difficult to use than a test based on peripheral blood lymphocytes, it should at a minimum permit testing of the original observations of Kellerman, Shaw and Luyten-Kellerman on the relationship of AHH and lung cancer. It should also provide a test system against which more convenient systems can be judged. If no more convenient systems are found it will be usable for the other epidemiologic projects we planned.

Arrangements for collecting blood from cases and controls remain operative and there will be no trouble supplying Dr. Kouri with samples again once his test system is firmly established. If we need to use a fibroblast test, new arrangements will have to be made to collect specimens - we envisage no real problems in doing this.

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Detailed Progress

(a) Spectrophotofluorometer (SPF) assay for 3-hydroxybenzo(a)pyrene (3-HOBP)

As stated in our last report this aspect of the assay was essentially workable once we began to employ nitrogen purges of SPF cuvettes, etc. However, since we began to work with cell lines and our results became more reproducible we found that this part of the test system was itself producing significant variation due to traces of the acetone-hexane mixture being transferred into

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the SPF cuvette on the sides of the pipette used for the transfer of the final NaOH layer: we have corrected this problem by changing the SPF layout to accommodate the test-tube with the acetone-hexane plus NaOH layer and eliminating the necessity for a transfer to the special small cuvette.

(b) Experience with complete peripheral blood lymphocyte test system

Table 1 shows some results from samples we sent to Dr. Kouri. (A considerable number of samples produced no results due to problems with air shipment resulting in non-viability of cells received in Bethesda - these problems have now been sorted out.) It is clear from this table that the test system used has major problems.

Generally repeatability of split-samples (testing all done blind by Dr. Kouri with coded samples) is not too bad but every fourth or fifth subsample gives a wild result. For example, a blood sample from O.D. split four ways gave AHH inducibility of 4.7, 4.3, 4.1 and 1.6. The first three results are probably acceptable as repeats, but clearly the 1.6 value is not. Repeatability from one week to the next is considerably worse. The first split-sample from A.E. averaged out at about 3 whereas the second averaged out at about 11.

Results we obtained in doing the test in our own laboratory were if anything even more variable than Dr. Kouri's.

A number of possible reasons for this failure to successfully establish the test system can be given. In particular, one may

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ask whether the PHA activation of the lymphocytes is just too variable.

Dr. Kouri is continuing work to try to sort out these difficulties. We decided, however, that we would retreat to what we hope will be a more easily standardized test using cell lines.

(c) Assaying AHH inducibility in cultured cell lines

Lymphocytes in vivo probably do not use their AHH enzyme system to any great extent and this may account for the extremely low amounts of AHH detected in 3MC induced lymphocytes in vitro (the above test system). This is certainly one of the problems with the lymphocyte test.

We decided therefore to measure AHH activity both in the most accessible cell line system, viz. fibroblasts, in order to obtain an epidemiologically usable test, and in liver and other cell lines in the hope of finding a line with high levels of AHH activity. The latter, if obtainable, should enable us to sort out more easily the best method of inducing AHH activity, etc.

The Chang liver cell line unfortunately does not produce high AHH activity as we had hoped, but tests on it did show up some of the problems and variables that need to be sorted out even in a "uniform cell population" AHH assay system.

Results from our last two experiments with "Chang" liver cells were as follows:

Experiment #15 - Chang cells from culture flasks were removed by trypsin, pooled, counted and distributed in amounts varying

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from 0.4×10^6 - 10.0×10^6 cells into 12 new flasks as shown in Table 2. Culture media was added to obtain a fixed volume of 10ml; 100λ 3MC was then added in the standard lymphocyte assay manner and the cells induced for 24 hours. The cells were then trypsinized, counted and assayed for AHH activity in the standard manner using BP for 45 minutes. The AHH activity (in SPF units) is given in the table (these results multiplied by 24 give a rough estimate of activity in picomoles).

Experiment #16 - Same procedure as for Experiment #15 done 1 week later. Results given in Table 3.

The results of both experiments are plotted in Figures 1 and 2. Other experiments on Chang cells at an initial number of 0.8×10^6 per flask have shown that the BP for zero minutes baseline control gives readings around 0.18 (in SPF units). Using this as the zero cells intercept (an assumption now being tested by running zero time controls with other cell numbers) we see that we may tentatively draw the conclusion that induction is probably linear up to 2×10^6 cells per flask and reproducibility week to week is probably achievable with this system. One problem is removing all cells from the flasks after induction (we are currently experimenting with Petri dished in a CO_2 atmosphere). Another is measuring substrate mass (e.g. should one use number of cells, quantity of protein or, as in certain mouse experiments, the weight of tissue? We suspect that conventional cell counting introduces a not insignificant amount of variability.

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Experiments with a fibroblast line ('1609') show a number of differences from these results with Chang cells. The peak SPF reading is obtained at a cell number of 1.2×10^6 cells and rather than simply flattening off when the cell numbers exceed this it drops sharply off. The peak SPF reading is however more than twice as high as Chang cells.

We are therefore concentrating on the '1609' cell line at present (and growing up other fibroblast lines for further work). On the basis of our work to date we presume that the "best" test system will be:

(1) Cultivation of cells in flasks.

(2) Removal with trypsin 72 hours before 3MC induction into Petri dishes

(3) Petri dish induction with 100λ 3MC at a cell number of 10^6/dish.

(4) Removal with trypsin and tube incubation with 100λ BP for 45 minutes.

Possible modifications requiring testing are:

(a) Mechanical handling of cells: does trypsin damage the cells? Would we do better at step (4) with a rubber policeman?

(b) Cell substrate measurement: would we get more repeatable results if we measured protein mass instead of number of cells.

(c) Is 3MC the 'best' inducer? (There is some evidence that 3MC does not induce monocytes.) How much 3MC should we use?

(d) Does the amount of BP added have any effect?

When these questions are answered there will be a number of possible ways to go, chosing priorities will depend on the progress other laboratories have made in the intervening period.

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(i) Apply the knowledge of optimum system for fibroblasts to other human tissues (including PHA stimulated peripheral blood lymphocytes) which do not require establishing cell lines.

(ii) Test a limited number of lung cancer cases and controls using fibroblast cell lines. This will be a lengthy procedure.

(iii) Test other cell line types for AHH inducibility to gain information on metabolism of hydrocarbons in different tissues.

If the stimulated lymphocyte test is established by Dr. Kouri (or any others) we will, of course, pursue as first priority our original plan of testing cases of cancer at various sites and controls.

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Table 1
Results of Specimens Sent to Dr. Kouri

Name	Date	AHH#	Constit.	Induced	I/C
A.E.	10/10	116	.96	2.2	2.3
	10/10	117	1.0	3.05	3.1
	10/10	119	.51	2.0	3.9
	10/10	121	.44	1.64	3.7
	10/24	153	1.0	14.0	14.0
	10/24	154	1.91	17.2	9.0
	10/24	156	1.35	9.7	7.2
	10/24	158	.84	10.0	11.9
	1/ 8	228	.022	.44	20.0
	1/ 8	229	.047	.34	7.2
	1/ 8	232	-----	-----	no growth
	1/ 8	233	.022	.18	8.2
A.A.	12/11	206	.094	.42	4.5
	12/11	207	.1	.41	4.1
	12/11	208	.099	.58	5.9
	12/11	209	.099	.47	4.7
A.R.	10/ 2	74	6.9	23.3	3.4
	10/ 2	75	2.5	10.0	4.0
	10/ 2	88	2.25	5.4	2.4
	10/ 2	90	1.42	4.2	3.0
	10/ 9	105	1.2	3.4	2.8
	10/ 9	108	0.60	2.4	4.0
	10/ 9	112	1.10	3.4	3.1
	10/ 9	113	1.25	4.0	3.2
A.J.	10/ 9	103	.56	3.15	5.6
	10/ 9	107	.62	2.72	4.4
	10/ 9	109	.43	2.78	6.5
	10/ 9	111	.885	3.9	4.4
B.J.	1/ 8	226	0.35	1.32	3.8
	1/ 8	227	----	-----	no growth
	1/ 8	230	0.74	1.85	2.5
	1/ 8	231	0.35	1.80	5.1
C.J.	8/21	11	1.68	4.52	2.7
	10/17	134	-----	-----	no growth
	10/17	137	-----	-----	no growth
	10/17	139	-----	-----	no growth
	10/17	141	-----	-----	no growth
	12/11	202	.075	.134	1.8
	12/11	203	.032	.136	4.3
	12/11	204	.065	.250	3.9
	12/11	205	.065	.220	3.4

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Name	Date	AHH#	Constit.	Induced	I/C
Chu. M.	10/ 2	77	3.5	7.0	2.0
	10/ 2	78	0.77	1.32	1.7
	10/ 2	89	0.52	.78	1.5
	10/ 2	91	0.61	1.96	3.2
	10/ 9	102	0.5	1.9	3.8
	10/ 9	104	0.35	1.0	2.9
	10/ 9	106	0.52	1.71	3.3
	10/ 9	110	-----	-----	no growth
	11/ 7	188	-----	-----	no growth
	11/ 7	189	-----	-----	no growth
	11/ 7	192	.57	3.55	6.2
	11/ 7	193	.56	3.1	5.5
Cof. G.	10/ 3	92	-----	-----	no growth
	10/ 3	93	-----	-----	no growth
	10/ 3	95	-----	-----	no growth
	10/ 3	99	-----	-----	no growth
Cof. M.	10/30	162	3.6	8.3	2.3
	10/30	163	3.9	8.9	2.3
	10/30	165	2.55	5.8	2.3
	10/30	167	2.96	7.33	2.5
	2/12	214	.11	.37	3.4
	2/12	215	.16	.495	3.1
	2/12	216	.09	.356	4.0
	2/12	217	.122	.71	5.8
D.A.	10/23	144	.39	1.18	3.0
	10/23	147	1.15	3.8	3.3
	10/23	151	1.4	4.9	3.5
	1/ 9	234	.078	.40	5.1
	1/ 9	236	.040	.13	3.3
	1/ 9	238	.06	.35	5.8
	1/ 9	240	.05	.183	3.7
H.M.	10/ 3	94	-----	-----	no growth
	10/ 3	96	-----	-----	no growth
	10/ 3	97	-----	-----	no growth
	10/ 3	98	-----	-----	no growth
	10/24	152	.88	4.7	5.3
	10/24	155	1.16	6.3	5.4
	10/24	157	1.31	5.1	3.9
	10/24	159	.76	4.9	6.4
	11/ 7	190	-----	-----	no growth
	11/ 7	191	-----	-----	no growth
	11/ 7	194	.95	5.6	5.9
	11/ 7	195	1.2	5.2	4.3

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Name	Date	AHH	Constit.	Induced	I/C
H.R.	10/10	114	-----	-----	no growth
	10/10	115	.42	2.5	6.0
	10/10	118	.58	2.5	4.3
	10/10	120	.45	1.66	3.7
M.L.	10/23	145	1.22	6.9	5.7
	10/23	146	.54	4.6	8.5
	10/23	148	.42	6.3	15.0
	10/23	150	.53	4.0	7.5
N.M.	1/ 9	235	.071	.31	4.4
	1/ 9	237	.073	.30	4.1
	1/ 9	239	.089	.498	5.6
	1/ 9	241	.094	.32	3.4
O.D.	10/31	170	.78	1.24	1.6
	10/31	172	.54	2.56	4.7
	10/31	174	.88	3.6	4.1
	10/31	176	.88	3.8	4.3
P.M.	8/21	8	3.15	10.91	3.5
	10/ 2	81	3.0	10.0	3.3
	10/ 2	82	5.2	10.7	2.1
	10/ 2	84	2.9	7.9	2.7
	10/ 2	86	4.5	9.0	2.0
S.M.	10/31	171	.44	2.75	6.3
	10/31	173	.42	2.65	6.3
	10/31	175	.64	1.15	1.8
	10/31	177	.51	2.30	4.5
S.A.	8/21	9	1.88	2.67	1.4
T.R.	10/30	164	1.64	4.6	2.8
	10/30	166	2.25	6.75	3.0
	10/30	168	2.5	7.7	3.1
	10/30	169	2.0	5.15	2.6
	12/12	210	.093	.41	4.4
	12/12	211	.11	.75	6.8
	12/12	212	.14	.5	3.6
	12/12	213	.16	.55	3.4

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Table 2
Experiment #15
Chang Cells induced with 100λ MC

Flask #	# cells (10^6) at 0 hrs.	# cells (10^6) at 24 hrs.	SPF readings
1	.4	.465	.274
2	.6	.855	.344
3	.8	.855	.329
4	1.0	.810	.407
5	1.2	1.73	.410
7	1.6	2.04	.514
8	1.8	1.80	.529
9	2.0	2.43	.380
10	4.0	3.92	.459
11	8.0	5.55	.555
12	10.0	4.19	.578

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Table 3
Experiment #16
Results with Chang Cells induced with 100λ MC

Flask #	# cells (10^6) at 0 hrs.	# cells (10^6) at 24 hrs.	SPF reading
9	.4	.289	.238
10	.6	.525	.307
12	.8	.705	.341
13	1.0	1.26	.337
14	1.2	1.43	.391
15	1.4	1.39	.474
16	1.6	1.72	.542

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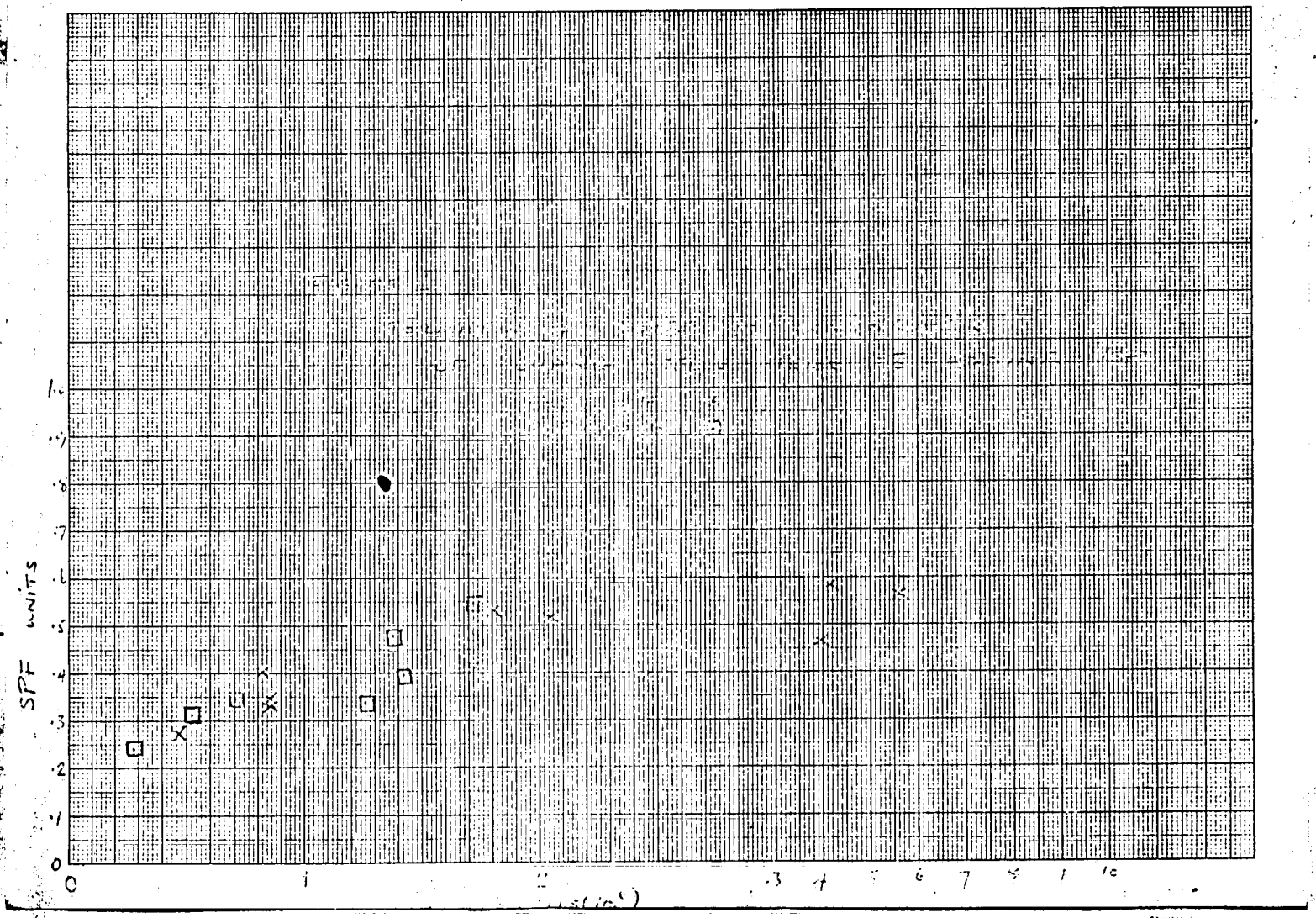
Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

K·E 10 X 10 TO THE CENTIMETER 18 X 25 CM. KEUFFEL & ESSER CO. MADE IN U.S.A. 46 1512



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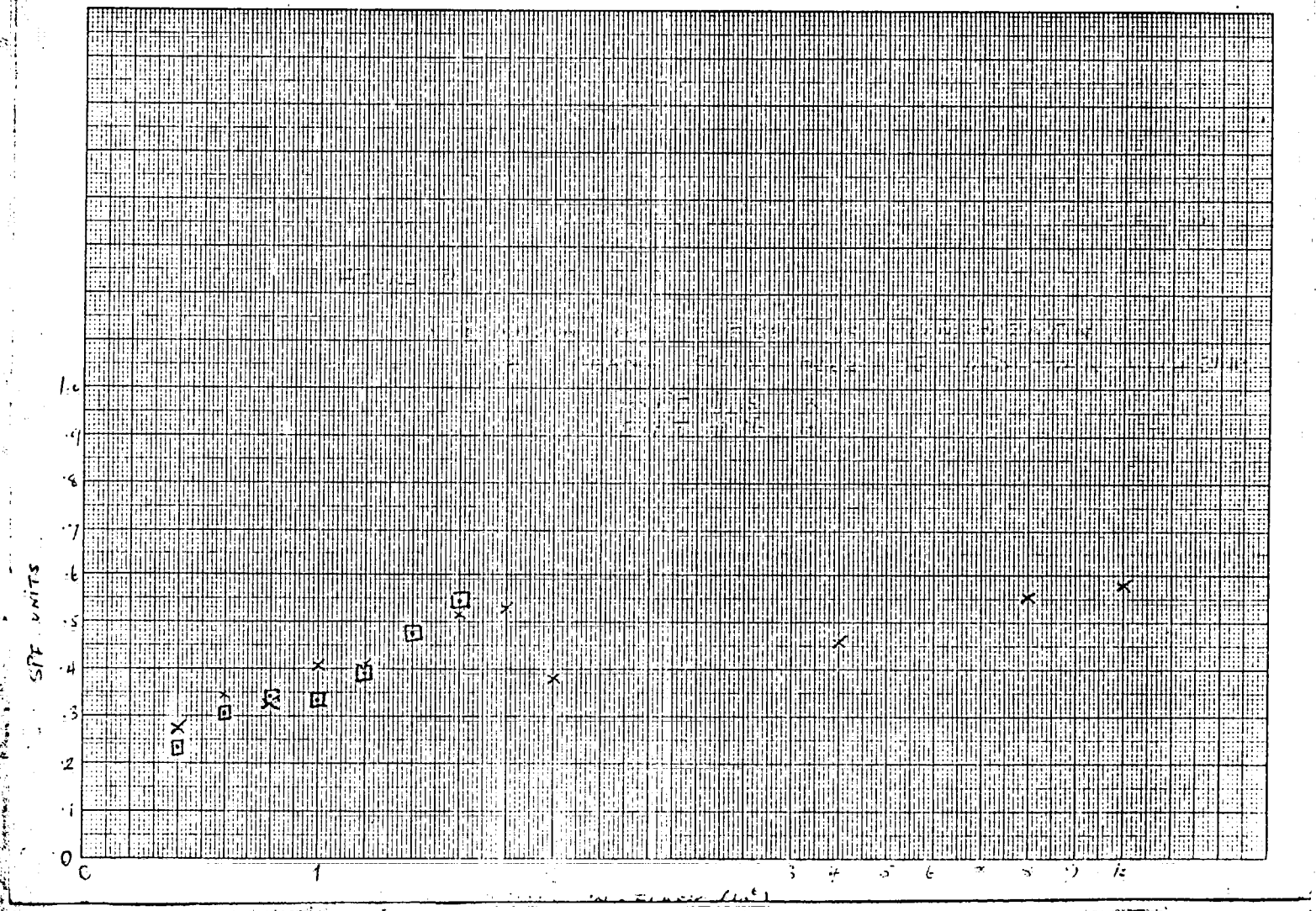
Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

K•E 10 X 10 TO THE CENTIMETER 18 X 25 CM. KEUFFEL & ESSER CO. MADE IN U.S.A.

46 1512



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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

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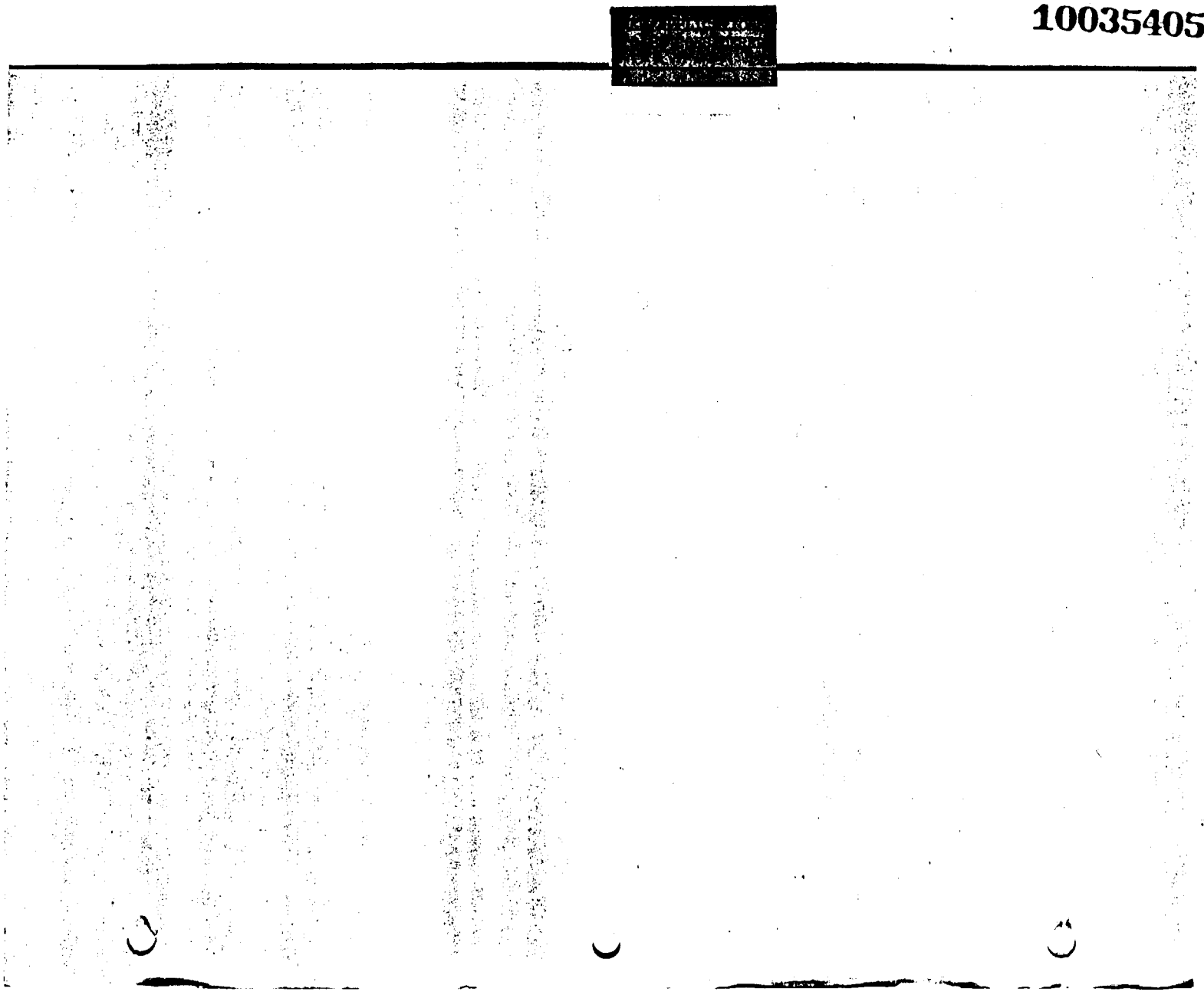

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

NEW APPLICATIONS TO BE CONSIDERED

Applic. No.	Principal Investigator	Amount Requested	Current Grant	Committee
CANCER				
1034	Castro	$ 46,383.00		Huebner, Jacobson, Sommers
945A	Ioachim	$ 30,000.00	$ 44,000.00	Feldman, Gardner, Huebner
573D	Loosli	$ 49,274.00	$ 60,000.00	Gardner, Jacobson, Meier, Sommers
891A	Neurath	$ 49,680.00	$ 46,120.00	Gardner, Huebner, Sommers
1005A	Wang	$ 47,163.00	$ 65,926.00	Huebner, Jacobson, Lynch
1031	Wattenberg	$ 90,707.00		Gardner, Huebner, Meier
CARDIOVASCULAR				
1032	Stein	$ 29,900.00		Bing, Gardner, Sommers
EPIDEMIOLOGY				
1037	Friberg	$ 32,919.00		Gardner, Jacobson, Lynch Sommers
PHARMACOLOGY				
1047	Aceto	$ 39,808.00		Bing, Jacobson, Sommers, Wyatt
836B	Essman	$ 71,274.00	$ 61,254.00	Bing, Gardner, Jacobson
1029	McKennis	$ 40,396.00		Bing, Jacobson, Sommers
1035	Neurath	$ 53,130.00		Bing, Huebner, Jacobson, Wyatt
PULMONARY				
1048	Bedrossian	$ 20,665.00		Gardner, Lynch, Sommers
1044	Buist	$ 22,561.00		Gardner, Jacobson, Lynch Wyatt
1043	Evans	$ 35,357.00		Gardner, Jacobson, Lynch Wyatt
1033	Feinstein	$ 24,883.00		Gardner, Sommers, Jacobson, Wyatt
1038	Guarneri	$ 27,897.00		Bing, Gardner, Jacobson
1041	Lauweryns	$ 32,420.00		Feldman, Gardner, Sommers, Wyatt

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Applic. No.	Principal Investigator	Amount Requested	Current Grant	Committee
Pulmonary - continued				
1050	Turino	$105,663.00		Gardner, Jacobson, Meier
1036	Will	$ 24,418.00		Bing, Jacobson, Sommers
MISCELLANEOUS				
1039	Kullander	$ 26,040.00		Gardner, Jacobson, Sommers
1040	Pierce	$ 45.339.00		Feldman, Gardner, Huebner, Wyatt
1030	Unanue	$ 63,490.00		Gardner, Feldman, Sommers
1042	Yoshinaga	$ 33,120.00		Bing, Gardner, Meier
DEFERRALS				
1021	Filley	$ 43,552.00		Jacobson, Liebow, Sommers
758C	Meier	$ 24,973.00		Feldman, Huebner, Jacobson
GEORGETOWN UNIVERSITY				
1049	Hamosh	$275,495.00		Feldman, Gardner, Jacobson
1045	Herscowitz	$ 46,675.00		Feldman, Gardner, Huebner, Jacobson
936R2	Vidic	$ 56,736.00		Gardner, Sommers, Meier, Wyatt

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CANCER

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

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#1034-CASTRO

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 17, 1975

Grant Application No. 1034

CANCER

TO: The committee comprising Drs. Huebner, Jacobson and Sommers

SUBJECT: Albert Castro, Ph.D., University of Miami School of Medicine, Miami, Florida
New application No. 1034
"Radioimmunoassay of Polyamine Markers in Cancer"

History

The new application No. 1034 was not handled as a case.

Castro is a current CTR grantee on a grant (No. 884M), approved by the SAB in March 1974, and entitled "Nicotine in Blood: Detection by Radioimmunoassay". A request for support ($46,383) for the third year of this study, covering the period January 1, 1975 through December 31, 1975, (application No. 884MR2) has been received by CTR.

Request

Application No. 1034 requests $44,558 for the first year of a three year project: estimates for the second and third years are $41,969 and $45,394, respectively.

Documents Submitted

1. Application dated May 30, 1975 (17 pages)
2. CV^{s} of Drs. Castro and Malkus (15 pages)

David Stone

DS/lp
Encls.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A. INC.

110 East 59th Street
New York, N.Y. 10022

Application For Research Grant

Date: May 30, 1975

1. Name of Investigator(s): include Title and Degrees)

Albert Castro, Ph.D., Associate Professor of Pathology and Medicine
Director, Hormone Research Lab
University of Miami School of Medicine

Herbert Malkus, Ph.D., Assistant Professor of Pathology
Director, Clinical Chemistry
University of Miami School of Medicine
Jackson Memorial Hospital

2. Institution & Address:

University of Miami
School of Medicine
Department of Pathology
P.O. Box 520875, Biscayne Annex
Miami, Florida 33152

3. Short Title of Project: RADIOIMMUNOASSAY OF POLYAMINES MARKERS IN CANCER

4. Proposed Starting Date: January 1, 1976

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Description of Objectives or Specific Aims:

The major objective of this proposal is to develop means of quantifying polyamines in special spermidine, spermine and putrecine as a marker in different types of malignant growth in animals and humans. In view of the low levels of the substance to be measured, and in view of our experience in detecting hormones, peptides and drugs, we believe that radioimmunoassay is the procedure of choice. The research proposal covers the development of specific antiserum against spermidine, spermine and putrecine. This methodology will be applied to measuring

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the levels of aliphatic polyamines, specific to patients with cancer of the lung, mammary and genital-urinary tract.

7. Give a Brief Statement of your Working Hypothesis: Appended

8. Details of Experimental Design and Procedures: (Attach Separate Pages) Appended

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Appended

10. Additional Requirements:

Freezer to store biological material

Refrigerated centrifuge

Animals to produce antibodies. We will require rabbits and goats.

11. Biographical sketches of all principal and professional personnel (append)

Appended

12. List of publications: (Five most recent as pertinent)(append)

Appended

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R: REDACTED MATERIAL

13. Budget: (1st year)

		% time	Amount
A.	Salaries (Personnel by names)		
	Professional		
	Albert Castro, Ph.D., Principal Investigator	20%	REDACTED
	Herbert Malkus, Ph.D., CoPrincipal Invest.	30%	
	Technical		
	Laboratory Technician	100%	8,164
	Retirement 8.95%		2,247
	Fringe benefits - 5% for faculty 847		
	7% for Staff 571		1,418
	Sub-Total		28,771
B.	Consumable Supplies (list by categories		
	Animals: 50 rabbits at $10 ea.		500
	maintenance $0.30/day/rabbit, ave. 30 days		450
	5 goats at $70 ea.		350
	maintenance $1.50/day/goat, ave. 90 days		675
	Chemicals and glassware		1,000
	Radioactive isotopes		1,500
	Sub-Total		4,475
C.	Other Expenses (itemize)		
	Travel to Scientific Meetings, 1 trip		500
	Sub-Total		500
D.	Permanent Equipment (itemize)		
	PJR-2 refrigerated centrifuge with attachments		4,500
	Freezer		500
	Sub-Total		5,000
E.	Overhead (15% of A + B + C)		5,812
	Total		44,558

Estimated Future Requirements:

	Salaries	Consu.Suppl.	Other Exps.	Permanent Exps.	Ovhd.	Total
Year 2	31,073	4,922	500	0	5,474	41,969
Year 3	33,559	5,414	500	0	5,921	45,394

It is understood that the application and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature [signature]
Director of Project 305/547/6508

Signature
Business Officer of the Institution
305/371/5572-Telephone

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

All current grants - $30,000 CTR - Radioimmunoassay of Nicotine

Pending -0-

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7. Working Hypothesis

Aliphatic polyamines such as spermidine, spermine and putrescine are widely distributed in nature. Their specific and vital physiological function as growth factors for certain microorganisms is emerging. The strong affinity that polyamines have for nucleic acid is attributed to neutralization of the phosphate group on the polynucleotides. Polyamines are naturally available intracellular stabilizers of biomembranes in many in vitro systems through formation of polyamine-phospholipid complexes.

Recently the spermine levels in urine in malignancy and pregnancy have been determined. The assays used in most of these studies were relatively insensitive and nonspecific. While their results are important, they should be re-evaluated with new, more precise methodologies to confirm their findings before the ultimate significance of polyamines as regulators of growth in neoplastic tissue is assigned. We propose for the determination of polyamines in biological materials on the basis of our previous experience the production of specific aliphatic polyamines antibodies and their use in the development of highly specific and sensitive radioimmunoassay.

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Background

Polyamines and cancer growth. Spermine and spermidine are present in all nucleated animal cells studied to date (1, 2, 3). In some rat tissues the spermidine-spermine ratio declines with the age of the animal (1, 4). The steady state concentration of putrescine in most mammalian tissue is almost always much lower than those of spermidine and/or spermine (3).

Even though the studies of the polyamines and their biological function increased during the last decade, not too much systematic attention has been given to polyamines and their biosynthetic enzymes in malignant cells. Significantly, Williams-Ashman et al (5) reported that in the most rapidly growing cell lines of the Morris hepatoma the concentration of putrescine were 5-10 times higher than those observed in the control liver. An increase in spermidine concentration with increased tumor growth rates were found to occur. The activity of ornithine decarboxylase was also noted to be significantly increased in all of the tumor studies and that continued synthesis of aliphatic polyamines in malignant cells occurred despite very rapid protein biosynthesis for other functions.

Spermine and spermidine have been shown to have potent inhibitory activity against neoplastic cells in vitro in the presence of calf serum containing spermine oxidase activity (6). 1003540540

Israel et al (7) found that some analogues of spermine and spermidine have shown significant in vitro anti-tumor activity against transplantable mouse tumors and also against human epidermoid carcinoma cells in culture systems containing calf serum.

Thirty-two analogues of spermine and spermidine were tested for significant anti-tumor activity. One analogue of spermine, a hydrochloride salt containing three methylenes between two secondary amino

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

groups in the molecule, and three spermidine analogues containing on one side an aliphatic chain 3,5, or 9 methylenes long, were found to be active. Later the same authors proved that the toxic activity of these analogues is again ascribed to their oxidation products, especially dialdehyde. Quash et al reported that in animal tissues beta-amino-propionaldehyde can arise directly from the oxidation of spermidine or diaminopropane. They found that the serum level of the bound aldehyde was markedly elevated in patients with malignant metastasis. In contract the serum level of bound aldehyde in patients with nonmetastastic malignancy was significantly lower than compared with patients with either non-malignant or malignant diseases who had responded to treatment (8,9). It is of particular importance that β-aminopropianaldehyde can be found in animal tissue from the enzymatic oxidation of spermine and that the product is a constituent of normal human serum. On the basis of these findings Quash proposed that the lowering of β-amino-propianaldehyde (BAPA) levels could be caused by partial block in the reaction leading from spermidine to BAPA. The decreased substrate levels of BAPA thus could lead to a corresponding drop in the product malandialdehyde. Hence, more rapid growth takes place. If the reaction leading from BAPA to maloandialdehyde is now itself inhibited, then the small amount of BAPA which has been fed through this pathway can no longer be oxidized. Therefore, an increase in BAPA occurs.

In spite of the above observations Tabor and Tabor (10) make it clear that it is still not possible at this time to categorically state that the apparent relationship between polyamines and nucleic acids is actual. Polyamines do have an essential role in vivo but it will be necessary to clarify which of the many observations are really related to the physiological functions of the polyamines.

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Polyamines and their Presence in blood and urine. The presence of spermine in the serum in malignancy has been demonstrated (10). Spermine was found in the formed elements of normal human cells, principally in leucocytes, but none free in the plasma (11). Raina (12) found that spermine and spermidine were present in the whole blood but not in the plasma under ordinary circumstances. Methods for determinations of these polyamines have been superseded by our advancing technology, and deserve re-evaluation. Shiman (13) found that spermine occurred at high concentrations in red blood cells in vertebrates other than mammals but that concentrations were very low in iron-nucleated mammalian red blood cell lines.

Kosaki et al (14) isolated from malignant tissue a phospholipid called "malignolpin", which has been reported to contain spermine and to also be present in the sera of some cancer patients. Recently Quash (15, 16, 17), using anti-spermine antibodies, reported that serum from patients with cancer and chronic infections precipitate directly with antispermine antiserum, while those from patients with other pathological conditions give negative reactions. This antigenicity in cancer patients appears to be directly reacting with anti-spermine antibodies and migrates in the region of the -lipoproteins and contains spermine. Its characteristics suggest a similarity to "malignolpin". Quash postulated that when a new methylation pattern is imposed any time on a cell, as may occur with certain viral infections or certain alkylating carcinogens, the cells react by overproducing a lipid component which is very acid for spermine, and which as a result has the lipospermine complex in them found in the serum of cancer patients. The Quash hypothesis is sophisticated and disciplined and deserves further effort to confirm and enlarge. Quash (18) also found in the study of the effect of antipolyamine antibodies on mammalian cells and tissue cultures, that these antibodies in the presence of a complement

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exerted a cytolytic effect on cells, and the morphological changes were associated with an increase of lysosomes. A mechanism to explain the increased lysosome content of cells having antibodies fixed to their surface is not evident. But according to Quash it may be that these lysosomes are involved in excision of blocked sites caused to make them susceptible to repair.

Spermine and spermidine or their acetyl derivatives have also been studied in urine. Nakajima et al (19) could not detect spermine and spermidine in the urine of normal children, but did consistently detect monoacetyl derivatives of spermidine. The presence of spermine, spermidine and putrescine in hydrolyzed urine of cancer patients was demonstrated by Russel et al (20) who found that patients with histologically diagnosed tumors and leukemias had several-fold greater values of polyamines in urine compared to the normals. Surgical removal of all or a portion of the tumor always leads to a decrease of polyamines in urine. Despite the encouraging studies to date, it has not been established that elevated urinary polyamines are associated with all kinds of human cancer.

Finally, because of problems of urine collection, renal clearance, renal amine oxidases and bacterial production of the polyamines, our thrust must be to individual plasma polyamine ratios against the background of total polyamines in a given sample.

Specific Aims

On the basis of previous findings we propose:

(a) A radioimmunoassay method be used to study the level of the polyamines, spermine, spermidine and putrescine in whole blood, plasma, and urine during normal growth in humans.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

(b) Study of the polyamine levels in cancer patients with different types of tumors during the development of the tumor and its treatment.

METHODS OF PROCEDURE

Two basic motivations will be involved in these investigations. First, elicitation of the minimum number of tests necessary to achieve an appropriate cancer detection and therapeutic monitoring program. Second, accumulation of information which will shed light on the three poorly defined pathways; spermine metabolism to the monoaldehyde; the glutathione conjugation; the acetylation pathways and their relationship to each other.

Production of Antibodies.

1) The carbodiimide procedure to produce antibodies to small molecules is one that has been used extensively (21). We have produced antibodies to many different small peptides and steroid molecules (22,23,24,25,26) by different conjugated antigens.

Aliphatic polyamines (spermidine, spermine, putrescine) will be conjugated to a macromolecular carrier (BSA or HSA) by a carbodiimide water soluble method using 1-ethyl-3-(-3-) dimethylaminopropyl)-carbodiimide Hcl or 1-cyclohexyl-3-(-2-mopholinyl-(4)-ethyl carbodiimide (27, 28).

The conditions of the reaction are as follows: The carrier (BSA or HSA), an excess of hapten, and reagents will be stirred together in aqueous solution for 30 minutes. The reaction is followed by dialysis and product isolation.

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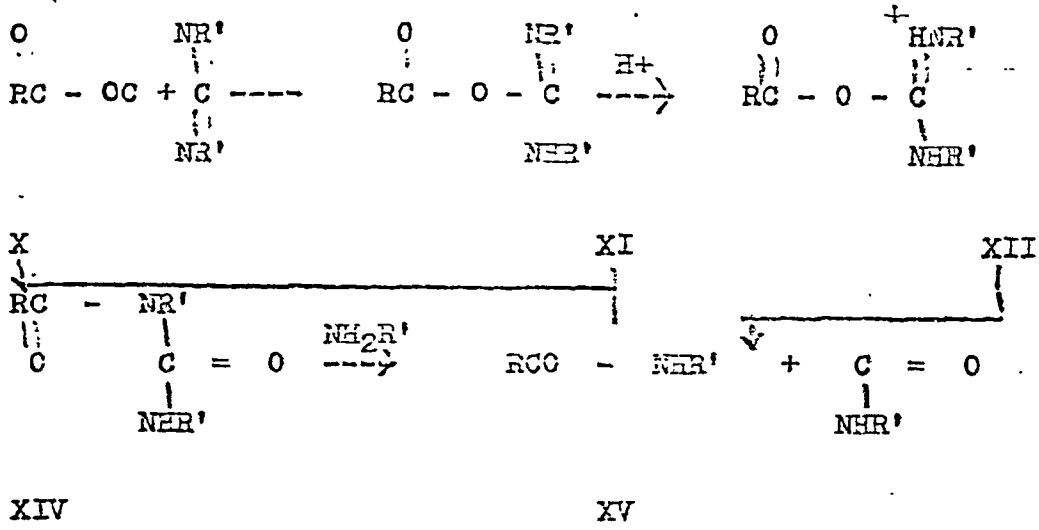
Reaction Mechanism



Reaction of carbodiimides with carboxylic acids

2) Conjugates obtained in this form will be injected into New Zealand white rabbits in 50% complete adjuvant via a) toe pad, b) intraperitoneal, 3) intramuscular.

Six weeks after the first injection, serum will be harvested, and by using labeled polyamines experiments on titer, specificity and affinity will be conducted.

Radioimmunoassay (Saturation analyses)

Radioimmunoassay is an extremely sensitive analytical method for detecting extremely low amounts of hormones, peptides, steroids, drugs and nucleotides. In practice this term has been applied to at least three methods whereby the use of a binding agent specific for the compound being determined is required, but these methods differ in the manner in which labeled compound is added and in the form of the calculations. In the other methods, or non-specific reactor or protein binding (CPB) one uses a non-specific protein with non-specific binding capacity to different molecules.

The two methods which will be used for development of radioimmunoassay for spermine and other aliphatic polyamines will be as follows:

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1) Add a known excess of binding agent to the solution containing the compound to be determined. The compound will take up some of the binding sites, but will leave the remainder free.

Add known amount of a highly specific labeled form of the same compound more than sufficient to saturate the remaining sites on the binding agent. Provided that the binding, once it occurs, is irreversible, then some of the radioactivity molecule will be bound and some will remain free. The ratio of free to bound molecules ($R \frac{F}{B}$) will be related to the number of sites on the binding agent which are taken up by the inactive molecules, that is, to the number of inactive molecules present. By calibrating the binding agent with known amounts of material the procedure can be made quantitative.

2) The other procedure involves mixing the compound to be determined and its labeled tracer before the binding agent is added. This means that instead of the inactive molecules being bound and the active molecule distributed between the remaining sites and the free state, the active and inactive molecules compete for the binding sites in equal basis. This does not require irreversible reaction between the molecule and the binding agent, and is in many ways similar to the substoichiometric dilution analysis.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

WORK ACCOMPLISHED BY US AND/OR OTHERS

A. We have in the last two years developed the majority of methods for radioimmunoassay in the literature, especially those related to hormones, peptides, steroids and drugs, and their application to clinical research studies. Our basic expertise lies in hooking up small molecules (low molecular weight) to many and varied macromolecular carriers by different chemical reaction techniques and producing specific high titer antibodies and developing highly sensitive specific radioimmunoassays for these compounds. (See publications in curriculum vitae.)

B. The detection of polyamines in body fluids has been of importance only recently, and because of the small quantities that are found, the techniques by which they have been measured are varied and non-specific.

1. The determination of polyamines in urine by gas-liquid chromatography/C.W. Gehrke, K.C. Kuo, R.W. Zumwalt, and T.P. Waalkes.
2. Gas chromatography-mass spectrometry (GC-MS) of diand polyamines in human urine; identification of monoacetylspermidine as a major metabolic product of spermidine in a patient with acute myelocytic leukemia/T. Walle.
3. Elevated polyamine levels in serum and urine of cancer patients: Detection by a rapid automatec technique utilizing an amino acid analyzer/L.J. Marton, J.G. Vaughn, I.A. Hawk, C.C. Levy, and D.H. Russell.
4. Clinical application of new methods of polyamine analysis/ M.D. Denton, H.S. Glazer, T. Walle, D.C. Zellner, and F. G. Smith.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Although polyamines have been studied for many years, until recently they have been of importance in fields related to growth. (Also see bibliography of grant application.)

1. Janne, J., Raina, A., Siimes, M.: Spermidine and spermine in rat tissues at different ages. Acta. Physiol. Scand. 62: 352-358, 1964.

2. Caldarera, C.M., Moruzzi, M.S., Rossoni, C., Barbiroli, B.: Polyamines and nucleic acids metabolism during development of chick embryo brain. J. Neurochem. 16: 309-316, 1969.

3. Bachrach, V.: Metabolism and function of spermine and related polyamines, Ann. Rev. Microbiol. 24: 109-134, 1970.

4. Russell, D.H., Medina, V.J., Snyder, S.H.: The dynamics of synthesis and degradation of polyamines in normal and regenerating rat liver and brain, J. Biol. Chem. 245: 6732-6738, 1970.

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9. Facilities Available

A. Space

The space will be comprised of 5000 sq. ft. with facilities for high and low levels of radioactivity, counting room, labeling room, conference room, and offices. This space will be shared.

The animal division is on the eighth floor of the Medical Research Building. Small animals (rats, guinea pigs and rabbits) are boarded in the Central University facility. Dogs will be kept in the facilities of the Division of Surgery.

B. Major items of equipment

Two liquid scintillation counters

One gamma counter

Actigraph strip counter

Gilford-Beckman recording spectrophotometer

Radiometer automatic titrator

Radiometer pH meters (2)

Mettler top pan balance

Sartorius analytical balance

Apparatus for paper and thin layer chromatography and electrophoresis

Vacuum oven

Two channel auto analyzers

Sowall RCB-2 centrifuge

Radioactive scanner

Hood for radioactive labeled sample drying apparatus

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4. Masayoshi, O., Inoue, H., Suzuki, F., Takeda, Y.: Studies on ornithine decarboxylase from the liver of thioacetamide-treated rats. Purification and some properties. Biochem. Biophys. Acta. 284: 285-297, 1972.

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8. Williams-Ashman, H.G., Coppoc, G.L., Weber, G.: Imbalance in ornithine metabolism in hepatomas of different growth rates as expressed in formation of putrescine, spermidine, and spermine. Cancer Res. 32: 1924-1932, 1972.

9. Israel, M., Zoll, E.C., Muhammad, N., Modest, E.J.: Synthesis and anti-tumor evaluation of the presumed sytotoxic metabolites of spermine and N,N'-bis (3-amino-propyl) nonane-1, 9-diamine. J. Med. Chem. 16: 1-5, 1973.

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11. Rosenthal, S.M., Tabor, C.W.: The pharmacology of spermine and spermidine. Distribution and excretion. J. Phar. Exp. Ther. 116: 131-138, 1956.

12. Raina, A.: Spermine and spermidine in normal human blood. Scand. J. Clin. Lab. Invest. 14: 318-319, 1962.

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14. Kosaki, T., Ikoda, T., Kotani, Y., Nakagawa, S., Saka, T.: A new phospholipid, malignolipin in human malignant tumors. Science 127: 1176-1177, 1958.

15. Quash, G., Makaraj, K.: Quantitative estimation of free and bound serum aldehyde. Clin. Chim. Acta 30: 13-16, 1970.

16. Quash, G.A., Wilson, H.B.: Polyamines in human serum. W.I. Med. J. 16:81-91, 1967.

17. Quash, G.A.: Immunochimie des polyamines. Compt. Rend. Acad. Sci. (Paris) 265D: 934-936, 1967.

18. Quash, G.A., Delain, E., Euppert, J.: Effect of anti-polyamine antibodies on mammalian cells in tissue culture. Expt. Cell. Res. 66: 426-432, 1971.

19. Nakajima, T., Zack, Y.F., Jr., Wolfgram, F.: N-mono-acetyl-spermidine as a normal constituent of urine. Biochim. Biophys. Acta. 184:651-652, 1969.

20. Russell, D.H., Levy, C.C., Schimpff, S.C., Hawk, I.A.: Urinary polyamines in cancer patients. Cancer Res. 31: 1555-1558, 1971.

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22. Castro, A., Kutas, M., Bartos, D., Chung, A.: A direct progesterone radioimmunoassay. Experientia 29: 636-637, 1973.

23. Castro, A., Grettie, D., Bartos, D., and Bartos, F.: LSD radioimmunoassay. Research Communications in Pathology and Pharmacology 6: 879-886, 1973.

24. Castro, A., Shi, T., Chung, A.: A direct plasma testosterone radioimmunoassay. Experientia 29: 1447-1448, 1973.

25. Cheng, L.T., Kim, S.V., Chung, A., Castro, A.: Amphetamines: New Radioimmunoassay. FEBS Letters 36: 339-342, 1973.

26. Castro, A., Bartos, D., Jelen, B., Kutas, M.: A radioimmunoassay for deoxycorticosterone in human peripheral plasma using sephadex LH-20 chromatography. Steroids 22 (6):851-867, 1974.

27. Sheehan, J., Cruickshank, P., and Boshart, G.: A convenient synthesis of water-soluble carbodiimides. J. Org. Chem. 26: 2525-2528, 1961.

28. Sheehan, J., and Hlavka, J.: The use of water-soluble and basic carbodiimides in peptide synthesis. J. Org. Chem. 21: 439-441, 1956.

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R: REDACTED MATERIAL

CURRICULUM VITAE

ALBERTO CASTRO, Ph.D.

Address: REDACTED

Birthdate:

Marital Status: REDACTED

Education and Degrees:

1953-1958 B.S., University of Houston, Houston, Texas

1957-1958 M.T., Jefferson Davis Hospital, Baylor University Houston, Texas

1959-1962 Doctor, Biological Chemistry, University of El Salvador, El Salvador, Central America

Postgraduate Education and Training:

1966-1968 Postdoctoral Research Fellowship in Diabetes and Hypertension: University of Oregon Medical School, Portland, Oregon.

1968-1970 Senior Research Fellow in Diabetes and Metabolism: University of Oregon Medical School, Portland, Oregon.

Aug. 1967 Radioisotopes in Medicine, in vitro Test, a course taken at the Clinical Laboratory, University of Oregon Medical School under E. Hine, M.D.

1963 Gas and Thin-layer Chromatography, a summer course given by the Department of Chemistry, University of El Salvador, under the direction of Professor J. Kannan, Ph.D.

Oct. 1964 Medical and Dental Curriculum, a course given by Leo Medelsky, Ph.D. of the University of Chicago at the University of Mexico.

Mar. 1965 Curriculum Integration in Medical Sciences, a course given by Leo Medelsky, Ph.D., of the University of Chicago at the University of El Salvador.

1965 Techniques of Teaching Science at Graduate Level, a course given by Faculty of Humanities, University of El Salvador.

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1966 Cultural Anthropology Applied to Biological Science, a summer course given by Armando Piovesan, M.D. of the University of Sao Paulo, Brazil.

Special Honors:

1964 General Relator, III International Congress of Dental Education for the Association of Latin American Dental Schools (120 Universities) Mexico.

1966 Award and Membership in ATENEO, Academy of Science of El Salvador.

Fellow, American Institute of Chemists

Northwest Pediatric Research

1966-1970 NIH Senior Research Fellow

Listed in:

American Men of Science--Physical and Biological Sciences
American Men and Women of Science--Medical Sciences
Who's Who in the West
Who's Who in the Southeast
Who's Who in Florida
Personalities of the West and Midwest
Community Leaders and Noteworthy Americans

Academic Appointments:

1958-1960 Assistant Professor of Microbiology and Biochemistry, Dental and Chemistry Schools, University of El Salvador, Central America.

1960-1963 Associate Professor, Biochemistry and Microbiology, Medical and Dental Schools, University of El Salvador.

1965-1968 Professor and Head, Department of Basic Sciences, Dental School, University of El Salvador.

1965-1966 Coordinator-Director, University of El Salvador General Studies and Pre-professional Curriculum.

1964-1968 Director of Research in Basic Science, Dental School, University of El Salvador.

1965-1966 Co-director of Graduate Research, Faculty of Chemistry, University of El Salvador.

1969-1973 Assistant Professor of Pediatrics and Co-director of the Pediatrics Metabolic Laboratory, University of Oregon Medical School, Portland, Oregon.

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1973-pres Senior Scientist, Papanicolaou Cancer Research Institute, Miami, Florida.

1972-pres Associate Professor of Medicine and Associate Professor of Pathology, University of Miami School of Medicine.

1974-pres Director, Hormone Research Laboratory, University of Miami, School of Medicine.

Experience in Academic Administration:

1963-1965 Member of the Following Technical Committees, Medical and Dental Schools, Admissions, Research, Graduate Education, University of El Salvador.

1961-1966 Member, Board of Directors, Dental School, University of El Salvador.

1963-1965 Member, University Supreme Council of Education, University of El Salvador.

1965-1966 Director of Curriculum Commission, Dental School, University of El Salvador.

Coordinator of expenditures for Scientific equipment in new science buildings from loans provided by International Branch of Development and other international organizations (for the Central American Universities).

1964-1965 Chairman, Research and Scholarship Committee, University of El Salvador

For many years I was assistant to the President of the University of El Salvador and Dean of the Medical and Dental Schools, and Consultant to other faculty deans in matters related to administrative and academic problems. I developed new educational methods and curriculum integration approaches which are now in use in many Latin American universities.

Professional Offices and Activities:

1967-1969 Member, Board of Directors of ATENEO, Academy of Science of El Salvador.

1967-1969 Member, Special Commission for Biological Sciences, ATENEO.

1965 Secretary, I Central American and II Costa Rican Congress of Microbiology..

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Professional Societies:

American Association of Medical Technologists

Sociedad de Microbiologia Latino Americana

Colegio de Quimica Biologica de El Salvador

Tooth and Bone Research Society, London

Fellow, Royal Society of Tropical Medicine and Hygiene, London

American Association for the Advancement of Science (AAAS)

American Association for Microbiology

American Chemical Association

New York Academy of Sciences

Fellow, American Institute of Chemists

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Publications:

1. Castro, A., Alfaro, A: Predominent microorganisms in the root canal in dental patients. Revista Sociedad Dental III: 23-29, 1962.

2. Castro, A.: Tuberculosis and deciclocerine. Revista del Sanatorio Nacional de El Salvador I: 219-223, 1963.

3. Mendez, E., Castro, A.: New approach for the treatment of root canal therapy. Anales of 4th Dental Congress of Central America and Panama, 1964, FOCAP.

4. Mackay, P., Castro, A.: Development of the tooth germ of rats in tissue culture. Anales of 4th Dental Congress of Central America and Panama, 1964, FOCAP.

5. Saca, M., Castro, A.: Tooth germ studies of reptiles in tissue culture. Facultad Odontologia II: 15-20, 1965.

6. Ugarte, E., Castro, A.: Potencia de la lizocima ante las cepas bacterianas mas comunes en conductor radiculares. Congreso Centroamericano I: 7 December, 1965.

7. Castro, A., Mendez, J.E., Sevilla, R.F., Beguer, P.M.: Poder bactericida de los cementos de obturacion de los conductos radiculares ante diferentes cepas bacterianas. Congreso Centroamericano de Microbiologia I: 6 December, 1965. II:9.

8. Castro, A.: Resistencia y Sensibilidad de la droga anti-tuberculosa Deciclocerina. Congreso Centro Americano de Microbiologia I: 16 December, 1965, II, 17.

9. Castro, A., Mendez, J.E.: Pasta poliantibioticas en endodoncia. Congreso Centroamericano de Microbiologia I: 17 December, 1965. II, 17.

10. Castro, A.: Organismos predominantes de los conductos radiculares. Congreso Centroamericano de Microbiologia I: 23 December, 1965. II,23.

11. Castro, A.: Algunas consideraciones sobre problemas de la educacion superior. Paper presented at the incorporation into ATENEO 3: 160, 1966.

12. Castro, A., Jovel, E.: "Areas Comunes", una nueva aplicacion a los estudios generales. Reforma Universitaria II: 1965.

13. Castro, A., Jovel, E.: Unidades valoratives and meritos y su aplicacion. Reforma Universitaria II: 1965.

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14. Castro, A., Jove, E., Escamilla, J.: Centralizacion de records academicos. University of El Salvador III: 1965.

15. Castro, A., Jovel, E.: Los guias y su importancia en la ensenanza de areas comunes. Universidad de El Salvador III: 1965.

16. Castro, A., Scott, J., Grettie, D., Macfarlane, D., Bailey, R.: Plasma insulin and glucose response of healthy subjects to varying glucose loads during three-hour oral glucose tolerance test. Diabetes 19: 842, 1970.

17. Bailey, R.E., Castro, A., Kramer, R., Macfarlane, D.: Enhancement of insulin release to acute glycemic stimulation with depression of basal insulin production rates in insulinoma following diazoxide administration. Acta Endocr. 63: 392, 1970.

18. Castro, A., Kline, W.B., Cyderman, W.G.: A semi-automatic device for dispensing absorbent materials in protein bound assay. Steroids 15: 641, 1970.

19. Buist, N.R., Campbell, R., Castro, A., and Brent, B.: Congenital islet cell adenoma causing hypoglycemia in the newborn. Pediatrics 605-610, March, 1971.

20. Price, L., Stone, G., Castro, A.: Perspectives in thyroid testing. Lab News 12-23, March, 1971.

21. Hammond, R., Dyess, K., Castro, A.: Insulin production and glucose tolerance in patients with granuloma annulare, Brit. j. of Derm. 87: 540, 1972.

22. Castro, A., Bartos, D., Bartos, F., Grettie, D., and Stone, G.: A practical guide in the diagnosis of hypertension. Lab News, 1972.

23. Bailey, R., Bartos, D., Bartos, F., Castro, A., Dobson, R., Grettie, D., Kramer, R., Macfarlane, D., and Sato, K.: Activation of aldosterone and renin secretion by thermal stress. Experientia 28: 159, 1972.

24. Castro, A., Bartos, F., Jowel, J., Grettie, D., Bartos, D., Stone, G., and Kondrasky, K.: A semiautomatic device for drying samples in steroid radioimmunoassay. Steroids 19: 59, 1972.

25. Castro, A.: Principles of competitive protein binding assay, ed. by W.D. Odell and W.A. Daughaday. Book Review. Lab Mangt. 10: 47, 1972.

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26. Alvarez, L.C., Dimas, C.O., Castro, A., Rossman, L.D., Vanderlaan, E.F., and Vanderlaan, M.P.: Growth hormone in malnutrition. J. Clin. Endocr. 34: 400, 1972.

27. Kutas, M., Chung, A., Bartos, D., and Castro, A.: A simple progesterone radioimmunoassay without column chromatography. Steroids 20: 697, 1972.

28. Castro, A.: Further experiences in islet cell adenomas. J. of Internat. Acad. Metabology 1: 5-24 (1), 1972.

29. Castro, A., Dyess, D.: Diabetes. Lab News 3: Dec., 1972.

30. Castro, A., Dyess, K., Buist, N., Kondrasky, K., and Potts, J.: The effect of diazoxide in children with islet cell adenomas. J. Clin. Biochem. 6: 211-219, 1973.

31. Chung, A., Kim, S.Y., Cheng, L.T., and Castro, A.: Phenobarbital specific antisera and radioimmunoassay. Experientia 29: 820, 1973.

32. Kendall, J., Allan, A., Jackson, B., Cook, D., Castro, A.: ACTH radioimmunoassay, Brain-Pituitary-adrenal interrelationship. Proceedings of a symposium, Cincinnati. A Brodish & W.S. Redgate (eds.), 1973.

33. Castro, A., Kutas, M., Bartos, D., Chung, A.: A direct progesterone radioimmunoassay. Experientia 29: 636, 1973.

34. Castro, A., Grettie, D., Bartos, D., and Bartos, F.: LSD radioimmunoassay. Research Communications in Pathology and Pharmacology 6: 879-886, 1973.

35. Castro, A., Jelen, B., Kutas, M.: A radioimmunoassay of Deoxycorticosterone. Experientia 29: 1581, 1973.

36. Castro, A., Shi, T., Chung, A.: A direct plasma testosterone radioimmunoassay. Experientia 29: 1447, 1973.

37. Cheng, L.T., Kim, S.Y., Chung, A., and Castro, A.: Amphetamines: New radioimmunoassay. FEBS Letters 36: 339-342, 1973.

38. Castro, A., Bartos, F., Bailey, R.E.: Angiotensin II antibody production. A comparative study. Biochem. Med. 8: 96-104, 1973.

39. Castro, A., Grettie, D.: Effect of diazoxide administration on plasma glucose and insulin secretion during two successive 100 gm glucose tolerance and IV tolbutamide. Biochem. Med. 9: 354-362, 1974.

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40. Allen, J.P., Cook, D., Greer, M., Paxton, H., Castro, A.: Evidence that acromegaly is not a hypothalamic disease. Transaction Assn. Amer. Phy. LXXXVI: 272-283, 1973.

41. Castro, A., Buist, N.R., Dyess, K.: The insulin-glucose ratio during hypoglycemia: A new phenomenon in the diagnosis of hormone producing tumors. Clin. Biochem. 7: 81-87, 1974.

42. Castro, A., Bartos, D., Kutas, M., and Weiss, G.: A new two-piece microcolumn in steroids separation for radioimmunoassay. Clin. Biochem. 7: 64-67, 1974.

43. Castro, A. and Hammond, R.: Glucose, insulin, HGH and cortisol plasma levels in granuloma annulare patient during glucose tolerance and cortisone glucose tolerance test. Clin. Biochem. 7 (2): 150-160, 1974.

44. Allan, J.T., Cook, D., Greer, A., Paxton, C., White, E., Castro, A.: Serial plasma GH concentration during selective removal of GH secreting pituitary tumors. J. Neurosurgery 41: 38-43, 1974.

45. Castro, A., Buist, N., Dyess, K., Bartos, F., Grettie, D.: Insulin values in normal pancreas and pancreatic adenomas in tissue extracts of children. Biochem. Med. 10: 208-213, 1974.

46. Castro, A., Chi, T., Chung, A.: A simple radioimmunoassay of plasma testosterone without column chromatography. Steroids 23 (5): 625-638, 1974.

47. Allan, J., Greer, M., MacGiebel, R., Swanson, R., Castro, A.: Endocrine function in an anencephalic newborn infant. J. Clin. Endo. Meta. 38: 94, 1974.

48. Castro, A., Bartos, D., Jelen, B., Kutas, M.: A radioimmunoassay for deoxycorticosterone in human peripheral plasma using sephadex LH-20 chromatography. Steroids 22 (6): 851-867, 1974.

49. Castro, A., Ugarte, E.: Human TBG solid phase radioassay. Res. Comm. in Chem. Path. & Pharma. 7 (3): 455-458, 1974.

50. Castro, A., Kutas, M., and Jelen, B.: A simultaneous measurement of aldosterone and deoxycorticosterone by radioimmunoassay. J. Steroid Biochem. 5 (1): 21, 1974.

51. Castro, A., Ugarte, E.: Vitamin B-12 and effect of pH changes on binding sites of different hog intrinsic factor in vitro assay. Res. Comm. in Clin. Path. & Pharmacology 8 (3): 411, 1974.

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52. Castro, A.: Clinical applications of RIA of drugs in humans. Medikon 7: 1974.

53. Castro, A., Bartos, F., Bartos, D., Grettie, D., Stone, G., Goe, D.: A practical guide to the diagnosis of hypertension. Lab News 3: 18, 1974.

54. Hoversland, A.S., Bingana, S., Castro, A., and Nelson, D.M.: Hematologic and serum biochemical parameters in lactating holstein cows following ingestion of aflotoxin-contaminated cottonseed meal. Press, J. of V. Sciences.

55. Harris, R., Hoversland, A.S., Nelson, D.D., and Castro, A.: Hematologic and serum biochemical parameters of the quarter horse: I. Changes from birth to twenty-six weeks of age. Press, J. of V. Sciences.

56. Castro, A., Kondrasky, K., Bartos, D., Bartos, F., and Hoversland, A.: Pygmy goat, thyroid function. Press, J. Lab. Anim.

57. Stephens, J.W. and Castro, A.: Lipoatrophic diabetes. Press, Diabetes.

58. Stein, R., Ugarte, E., Castro, A., Pires, G.: The clinical usefulness of TSH in the diagnosis of hypothyroidism in patients on thyroxine treatment. Press, J.A.M.A.

59. Dinnsa, B., Castro, A.: Comparative biochemical parameter of different deer species. Press, J. Vet. Sci.

60. Castro, A., Hoversland, A.S.: Electrolite levels in the pygmy goat. Press, J. Vet. Sci.

61. Fisher, G.W., Vasquez, A.M., Buist, N.R., Campbell, R., Egam, E.T., Castro, A.: Neonatal islet cell adenomas. Press, J. Pediatrics.

62. Castro, A., Schultz, D.R.: Comparative studies of the production of antibodies to angiotensin I. Press, Experientia.

63. Castro, A., Schultz, D.R.: Renin activity solid phase radioimmunoassay. Press, Res. Comm. in Clin. Path. & Pharm.

64. Metcalf, D., Bartos, D., Bartos, F., Castro, A.: Renin and aldosterone secretion in pygmy goats during progestin implant. Press.

65. Castro, A.: Comparative study of steroid antibody production in different animal species. Press, Steroids.

66. Allan, J., Cook, D., Greer, M., Castro, A.: Hormone patterns in plasma and CFS in active acromegalic patients. Press, Archives of Neurology.

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67. Stephens, J.W., Castro, A.: Growth hormone release in juvenile diabetes during acute stress. Press, Diabetes.

68. Gratzner, H.G., Leif, R.M., Castro, A.: The use of antibody for bromodeoxyuridine for immunofluorescent determination of DNA replication in single cells. Press, Experimental Cell Research.

69. Allen, J.P., Kendall, J.W., McGilura, R., Lamorena, T.L., and Castro, A.: Adrenocorticotropic and growth hormone secretion studies during pneumonencephalography. Arch. Neurol. 31: 325-328, 1974.

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RESEARCH MATERIAL PRESENTED IN ABSTRACT FORM

1. Bailey, R.E., Brice, L.T., Castro, A., Lockwood, D.: Juvenile mineralcorticoidism with adrenal hyperplasia (AH) - an unusual cause of hypertension. Northwest Society for Clinical Research, Vancouver, B.C., January, 1968. Proceedings 3rd. International Congress of Endocrinology, Mexico City, June, 1968.

2. Bailey, R.E., Brice, L.T., Castro, A., Lockwood, D.: Juvenile mineralcorticoidism with adrenal hyperplasma (AH) - an unusual cause of hypertension. Clinical Res. 16: 156, 1968, (Jan).

3. Castro, A., Scott, J., Brice, L., Belts, R., Kimberling, W., Bailey, R.E.: The plasma glucose (PG) response to different oral glucose loads in healthy subjects. III International Congress of Endocrinology, Mexico City, June, 1968. American Diabetes Association, 28th Annual Meeting, San Francisco, June, 1968. Diabetes 17: 319, 1968.

4. Bailey, R.E., Brice, L.T., Castro, A., Kramer, R., Macfarlane, D., Bartosova, D.: Periodicity in the "ectopic ACTH syndrome" a new finding. Clin. Res. 17: 140, 1969.

5. Castro, A., Scott, J.P., Grettie, D.P., Brice, L.T., Macfarlane, D., Bailey, R.E.: The dynamics of insulin production and blood glucose concentrations as a function of incremental oral glucose loads in man. Clin. Res. 17: 122, 1969.

6. Castro, A., and Grettie, D.: Effect of diazoxide administration on plasma glucose and insulin secretion during two successive 100 gm glucose tolerance and IV tolbutamide. VII Congreso Panamericana de Endocrinologia, Sao Paulo, Brazil, 1970.

7. Bailey, R.E., Bartos, D., Bartos, F., Castro, A., Dobson, R., Kramer, R., Macfarlane, D., Sato, K.: Activation of aldosterone production and the renin-angiotensin system by acute thermal stress. Endocrine Society Meeting, San Francisco, California, 1971.

8. Chung, A., Bartos, D., Bartos, F., Grettie, D., and Castro, A.: Radioimmunoassay of desoxycorticosterone (DOC). Clin. Res. 20: 175, 1972.

9. Alvarez, L.C., Dimas, C.O., Castro, A., Rossman, L.G., Vanerlaan, E.F., and Vanerlaan, W.P.: Growth hormone in malnutrition. Clin. Res. 20: 163, 1972.

10. Castro, A.: Application of radioimmunoassay testing to disease predictability, Ann. N. Am. Med. & Dental Assn. 5: 10, 1972.

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11. Castro, A., Buist, N.: Insulin/glucose ratio in diagnosis of islet cell adenomas in children. Clin. Res. XXI (2): 289, Feb., 1973.

12. Buist, N., Castro, A.: Protein induced hypoglycemia. Clin. Res. XXI (2): 315, Feb., 1973.

13. Allan, J.T., MacGiebel, R., Greer, A., Castro, A.: Endocrine function in the anencephalic man. Clin. Res. XXI (2): 248, Feb., 1973.

14. Hammond, R., Dyess, K., Castro, A.: Insulin production and glucose tolerance in patients with granuloma annulare. Dermatology Digest 7: 1973.

15. Gratzner, H.G., Leif, R.C., Castro, A.: Determination of DNA synthesis in cells by immunological methods, utilizing antibodies specific for bromodeoxyuridine. XI International Cancer Congress Panel 29, Cell biology 11, page 313, Florence, 1974.

16. Gratzner, G.H., Lief, R.C., Ingram, D.J., Castro, A.: Immunoflorescent identification of DNA replication in sigle cell VII. Miami Winter Symposia, 1975.

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R: REDACTED MATERIAL

CURRICULUM VITAE

HERBERT MALKUS, PH. D., Assistant Professor, Department of Pathology, University of Miami School of Medicine, and Director of Clinical Chemistry, Jackson Memorial Hospital, Miami, Florida, 1973 to present.

Date of Birth: REDACTED

Marital Status: REDACTED

Academic Background:

B.S., Brooklyn College, Brooklyn, New York, 1964.

Ph.D., Iowa State University, Ames, Iowa,
Teaching Assistant, 1964-1966
Research Assistant, 1966-1968

Postdoctoral Fellow, University of Florida, Gainesville, Florida, September, 1968 to March, 1970.

Interim Assistant Professor, University of Florida, Gainesville, Florida, 1970-1971.

NIH Postdoctoral Fellow Trainee, Clinical Chemistry, Yale University, New Haven, Connecticut, 1971-1973.

Research Experience:

Kinetics and mechanisms of the disproportionation of Iodosobenzene.

Electron spin resonance studies of semidiones derived from aliphatic acyloins

Synthesis of aliphatic acyloins

Synthesis of ^{13}C substituted aliphatic acyloins.

Synthesis of tetracyclododecadienes and bird cage compounds

Electron spin resonance studies of cyclopropyl conjugation

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Research Experience: Synthesis of platinum and palladium organometallic complexes

Thyroid Function Tests. Radioimmunoassay of triiodothyronine

Automated methods of analyses

Honors and Societies:
American Chemical Society, 1962
Sigma Xi, 1964
Phi Lambda Upsilon, 1966; Vice-President, Theta Chapter, 1967-1968
Alpha Chi Sigma, 1970
American Association of Clinical Chemists, 1973

Publications:

1. Russell, G. A., and Malkus, H.: Cyclopropyl Semidiones. J. Amer. Chem. Soc. 89: 160, 1967.

2. Chalfont, G. R., and Malkus, H.: Cyclopropyl Nitroxide Radicals. Abstract 158th National Meeting of the American Chemical Society, New York, N.Y., 1969, orgn 124.

3. Battiste, M. A., Haywood-Farmer, J., Malkus, H., Seidl, P., and Winstein, S.: J. Amer. Chem. Soc. 92: 2144, 1970.

4. Malkus, H., Battiste, M. A., and White, R. M.: Radical Anions Derived from Alkyl-substituted 1,2,4,5-Tetrazines. Chem. Communications, 479, 1970.

5. Russell, G. A., Lawson, D. R., Malkus, H. L., and Whittle, P. R.: ^{13}C-Hyperfine Splitting in Semidiones. J. Chem. Physics 54: 2164, 1971.

6. Haywood-Farmer, J., Malkus, H., and Battiste, M. A.: Synthesis and Nuclear Magnetic Resonance Spectra of Some Bridged Polyhydromethanonaphthalenes. Bridge Proton Assignments. J. Amer. Chem. Soc. 94: 2209, 1972.

7. Malkus, H., and Donabedian, R. K.: Triiodothyronine Radioimmunoassay. Clin. chim. Acta 51: 191, 1974.

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Papers in Preparation:

1. Malkus, H., Seligson, D., and Seligson, H.: Estimated Free Thyroxine, an Improved Measurement of Free Thyroxine.

2. Jatlow, P., and Malkus, H.: An Improved G C Assay for Phenobarbital, Primidone and Diphenylhydantoin.

Areas of Interest:

1. Radioimmunoassay of hormones and steroids

2. Thin layer chromatography of alkaloid metabolites

3. Automation of enzyme analyses

4. Thyroid Function Tests

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 23, 1975

Grant Application No. 945A

CANCER

TO: The committee comprising Drs. Feldman, Gardner and Huebner

SUBJECT: Harry L. Ioachim, M.D., Lenox Hill Hospital, New York
Continuation application No. 945A
"The Immune Response at the Tumor Site in Lung Carcinoma"

History

A plan entitled "Studies of Human Lung and Ovarian Carcinomas" was approved by the SAB in October 1973 at a level of $40,000. The first year renewal application (No. 945R1) requested $55,700 and was approved by the SAB in October 1974, in the amount of $44,000 for the period January 1, 1975 through December 31, 1975, without assurance of further support.

This continuation application (No. 945A) with change in title concerns lung and is not involved in work with ovarian carcinoma.

Request

Application No. 945A requests $30,000 for the first year of a three year plan: estimates for the second and third years are $33,000 and $36,300, respectively.

Documents Submitted (attached)

1. Application dated July 17, 1975 (27 pages, including CV's of Dr. Ioachim, Brent H. Dorsett and Edward P. Paluch).

2. Manuscript submitted for publication (41 pages including Figures and Tables).

Comment

Progress Report No. 2 (8/1/74 - 7/31/75) is due at CTR in the near future, and will be forwarded on to you as soon as received.

David Stone

DS/lp
Encls.

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945A

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 18 1975

Application for Research Grant
(Use extra pages as needed)

Date: July 17, 1975

1. Principal Investigator (give title and degrees): Harry L. Ioachim, M.D.
-Clinical Professor of Pathology, College of Physicians and Surgeons of Columbia University
-Attending Pathologist, Lenox Hill Hospital

2. Institution & address:
Lenox Hill Hospital
100 East 77 Street
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

The Immune Response at the Tumor Site in Lung Carcinoma

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

see appendix #1

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Appendix #1

Based on the hypothesis that the tumor site contains the highest concentration of tumor-associated antigens and includes the specifically-sensitized lymphoid cells as well as the tumor-directed antibodies that they produce, our research aims will be:

1. The characterization of reactive histologic patterns associated with various types of lung carcinomas.

2. The functional identification of lymphocytes and plasma cells of the stromal reaction of lung carcinomas (i.e. T-cells, B-cells, IgG-, IgM-, IgA-secreting cells, etc.).

3. The isolation of specific antibodies from solid lung carcinomas and their pleural effusions.

4. The extraction, purification and characterization of antigens associated with lung carcinomas.

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8. Brief statement of working hypothesis:

see appendix #2

9. Details of experimental design and procedures (append extra pages as necessary)

see appendix #3

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Appendix #2

Previous work in this (1-4) and other (5-10) laboratories indicates the existence of tumor-associated antigens in human cancer. The present search for antigens specific for different types of tumors is strongly stimulated by the potential application of such antigens in the early diagnosis of cancer. Similarly, the identification of specific antibodies is of great practical importance for their use in immunodiagnosis and immunotherapy.

At the present time, the investigation of specific antigens in cancer patients is based primarily on skin tests (11-13) and on assays of lymphocyte reactivity to tumor extracts and tumor cells (14-16). A sustained effort is also being made to detect circulating antigens and antibodies in sera of cancer patients and several tumor-associated antigens have been described (1-2, 5-10, 17-18). However, to date, most of these efforts have not yet produced the expected results, possibly because tumor antigens in the circulating blood are diluted to infinitesimal amounts or locked in antigen-antibody complexes. Similarly, the lymphoid cells specifically primed to react to a particular tumor are highly diluted in the pool of circulating lymphocytes.

Based on our earlier studies (4, 19), it appeared to us that the tumor site would be a more logical place to explore the presence of tumor-reactive populations of lymphoid cells as well as to attempt the detection of tumor-associated antigens and antibodies.

Thus, the working hypothesis of this project is that the tumor itself with its reactive stroma is the site where specific antigens and antibodies could be expected to be present in highest concentration

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Appendix #3

1. The characterization of reactive histologic patterns associated with various types of lung carcinomas.

Recent work from this laboratory (19) indicates that the stromal cellular reaction of lung carcinomas correlates with the histologic types and the degrees of differentiation of the tumors. Distinctive patterns of stromal cellular reaction, characteristic for different histologic types of lung carcinoma, were thus recognized. The amount of cellular infiltration was highest in squamous cell carcinomas and lowest or non-existent in oat cell carcinomas. Within the various histologic categories the well differentiated tumors appeared to be accompanied by more reactive cells than the poorly differentiated and there was no relation between tumor necrosis and cellular infiltration. The plasma cells were distinctly associated with squamous cell carcinomas and their amount in the stroma was proportionate to the degree of differentiation and the presence of keratin produced by the tumors.

Based on these observations we propose to undertake a study of clinical-pathologic correlations in which the patterns of stromal cellular reaction will be investigated and corroborated with the tumor type and grade as well as with the clinical history of the patients. To characterize the tumors we will consider their histologic diagnosis, degree of differentiation, amount of necrosis and capacity for invasion (vessels, lymph nodes, and pulmonary tissues). These features will be compared with the cellular reaction at the tumor site. The latter will be estimated according to several

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parameters: amount, composition and distribution of both cellular infiltrates and of fibrous components. Details of these criteria are given in a recent study on the stroma reaction of lung carcinomas (19).

These data will be correlated with the clinical information available in the history of the patients such as age, sex, time from first tumor symptoms, clinical stage, treatment and survival. The total count of lymphocytes in the peripheral blood and the number of T-lymphocytes will be also considered since it was shown in a recent study from this laboratory (22) that the levels of T-lymphocytes were significantly decreased in patients with breast cancer.

2. The functional identification of lymphocytes and plasma cells of the stromal reaction of lung carcinomas.

The presence of large numbers of plasma cells and lymphocytes in the stroma of lung carcinomas and their selective association with different types of tumors (19) suggests that specific antibodies might be released by these cells.

In our experience, when cryostat sections of lung carcinomas were stained with anti human immunoglobulins in direct immunofluorescence, the cellular infiltrates, plasma cells and lymphocytes appeared positive for various immunoglobulins, IgG, IgM and IgA. The eluates which we recovered subsequently from such tumors reacted positively in indirect immunofluorescence with tissue-cultured lung adeno and squamous carcinoma cells but not with cell sus-

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pensions of normal lung or tissue-cultured cells of various non pulmonary cancers.

In the present study we plan to attempt the identification of various types of lymphoid cells as well as the investigation of their secretory activity. Several techniques that have been recently set up in our laboratory will be used for this purpose. To determine if the reactive lymphocytes in the tumor stroma belong to the T or B classes of cells, lung carcinoma tissues will be minced and the resulting cell suspension will be separated into lymphoid cells and tumor cells with the aid of a modified Ficoll-Hypaque technique (30). The lymphoid cells thus separated will then be reacted with sheep erythrocytes (SE) to determine the numbers of T cells, as previously described (22) and with erythrocytes coated with antibody and complement (EAC) to determine the numbers of B cells (31).

To observe the location within the tumor tissues of T and B lymphoid cells we will also use the SE and EAC techniques on cryostat sections according to the methods described by Jaffe et al (31) which have already been used in our laboratory. The classes of immunoglobulins secreted by the lymphocytes and plasma cells of the stromal reaction will be determined by specific staining with fluorescein-labelled anti human IgG, IgM and IgA antisera and subsequent examination under ultra violet light (indirect immunofluorescence).

To permit the ultramicroscopical visualization of immunoglobulins on the surface of lymphoid cells we will employ a similar technique, previously used in our laboratory (32) by which ultra-thin sections of lung carcinomas will be reacted with peroxidase-labelled anti human immunoglobulins and examined under the electron microscope.

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3. Isolation of specific antibodies from solid lung carcinomas and their pleural effusions.

Results of work done in our laboratory over the last year strongly suggest the existence of tumor specific autologous antibodies in patients with carcinoma of the lung.

Antibodies eluted from solid lung tumors have reacted positively in the indirect immunofluorescence test with both autologous and allogeneic lung tumor cells while failing to react with other normal or malignant cells. The recovery of such autologous anti-tumor antibodies from pleural effusions have been successfully attempted with quantitatively superior results. It appears that certain malignant effusions in lung carcinoma may represent a rich source of tumor specific antibody although its apparent activity may be masked by immune complex formation with shed soluble antigens present in the fluid.

Antibodies derived from lung carcinoma pleural effusions have demonstrated at least partial cross reactivity between cells derived from both squamous cell carcinoma and adenocarcinoma of the lung, while failing to react with cells derived from oat cell carcinoma of the lung. Conversely, such antibody activity has been demonstrated for epidermoid and adenocarcinoma but not for oat cell carcinomas. The antibodies recovered from malignant pleural effusions have been tested for reactivity with known tumor associated antigens including CEA by the radioimmunoassay and found to possess no such reactivity.

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The present proposal projects the expansion of our findings according to the following plan.

Recovery of higher yields of tumor specific antibody from malignant effusions.

Although substantial yields of tumor reactive antibody (up to a titer of 1:64 in I.F.) have been prepared in this laboratory by acid disassociation and salt precipitation, results of preliminary experiments in this and parallel systems indicate that much higher yields of antibodies might be recovered. Recovery of significantly greater titers of antibody activity may allow the use of more informative but less sensitive analytic techniques such as double immunodiffusion. Such improved antibody recoveries can be obtained in two ways according to our experience:

a) Batch processing by ion exchange resins to prepare pure γ- globulin fraction (including antigen antibody complexes).

When large volumes of malignant effusions are received they are immediately dialysed to lower their ionic strength. They are then mixed with an amount of preswollen ion exchange resin capable of binding 2x the total protein content of the fluid and agitated for one hour. The ionic strength and pH of the slurry are adjusted to a range where only Ig G and Ig G complexes will elute from the gel and the liquid phase recovered by filtration. The resultant clear fluid contains only Ig G and is easily concentrated by ultrafiltration (20,21).

b) Separation of disassociated immune complexes on cation exchange resins at low pH.

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Concentrated Ig G, Ig G-antigen complex fractions are subjected to low pH to disrupt immune complexes and absorbed to cation exchange resin columns at low pH. The columns are then eluted at a pH slightly below the isoelectric point of Ig G. This elution removes all serum protein from the column including antigen liberated from immune complexes. The bound Ig G is then removed by adjusting the pH to above the isoelectric point of Ig G while minimizing the loss of antibody activity due to acid hydrolysis.

In all these procedures the quantity and purity of immunoglobulin fractions are ascertained by radial immunodiffusion and immunoelectrophoresis in 1.5% agarose (19). The specific reactivity of recovered antibodies is determined by indirect immunofluorescence tests as previously described (23,24) using both cell suspensions obtained directly from tumors and cells from tissue culture as antigen substrates.

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provide a simple method for evaluation of antigen recovery during purification.

Purification and characterization of antigens will be attempted by fractionation of crude tumor extracts using ion exchange chromatography combined with molecular sieve chromatography.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

see appendix #4

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

see appendix #5

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see appendix #6

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Appendix #4

Facilities Available

The Cancer Research Unit located at the 12th floor of the Lenox Hill Hospital includes 3 spacious, air-conditioned laboratories, 1 tissue culture room, 1 equipment room, and the electron microscopy laboratory.

The tissue culture room is fully equipped with ultra violet sterilizing lamps. sterile-work hoods, 4 CO_2-gassed incubators and 2 inverted microscopes for tissue cultures.

The immunology equipment includes:

1 Beckman ultracentrifuge

1 Sorvall centrifuge (Super Speed)

1 MSE Sonifier

2 Leitz Microscopes for Immunoflourescence with photo equipment

1 Revco deep freeze

1 Column chromatography set up, including a variety of experimental and preparative columns, a continuous flow U.V. monitor and recorder and a 200 tube automated fraction collector

1 Lyophilizer

1 Agarose immunoelectrophoresis equipment

1 Amicon 8 cell experimental ultrafiltration device

In addition our laboratory has in its inventory:

1 Liquid nitrogen

4 large freezers and refrigerators

1 Cryostat microtome

1 Three channel 100 sample automated Beta Counter

1 Three channel 200 sample automated Gamma Counter

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The electron microscopy laboratory includes one 101 Siemens and RCA electron microscopes, 2 ultramicrotomes and a photography lab.

There are also animal facilities perfectly adequate for several hundred laboratory animals, including mice, rats, guinea pigs and rabbits. A separate mating room has allowed us to raise our own colonies of inbred animals, decreasing substantially the need for purchase.

A full-time animal caretaker and a full-time research assistant preparing and sterilizing the glassware are being entirely salaried by Lenox Hill Hospital.

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Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Harry L. Ioachim	Clinical Professor	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED	REDACTED	REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
1. Lyceum "Cultura"-Bucharest	Baccalaureat	1943
2. Faculty of Medicine- Univ. of Bucharest	M.D.	1951
3. Dept. of Pathology- Faculty of Medicine Univ. of Bucharest	Instructor; Asst. Prof.	1951-
4. First Surgical Clinical- Faculty of Medicine. Univ. of Bucharest	Head of Path.	1951-
5. Institut du Cancer-Villejuif-Paris	Chargé de Recherches	1961-
6. Dept. of Path.-College of Phys.& Surg.-Columbia Univ	— Asst. Prof.	1962
	— Assoc. Clin Prof.	1968
	— Clin. Professor	1973-
7. Dept of Path. Lenox Hill Hospital	Attending Pathol.	1968-
8. New York State Board Licence	M.D.	1967
9. Board of Pathology	Pathologist	1967

HONORS

Career Scientist Award of the Health Research Council of N.Y.-1956
Awardee of National Cancer Institute-Grants in aid- 1967.1970.1973
Awardee of New York Cancer Research Institute.-Grant in aid-1970
Permanent Editor of "Pathobiology Annuals"-Appleton-Century-Crofts. publishers, first five volumes published: 1971-1975

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Tumor Immunology. Leukemia, Oncogenic Viruses	Principal Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Twenty five years of practice in pathology
Twenty years of activity in Experimental and Clinical Oncology
Ninety eight publications in Pathology and Oncology

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Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Brent H. Dorsett	Research Associate	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED		REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
Brooklyn College City University of New York	B.S. Biochemistry	1968

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Human Tumor Immunology	Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Ten years of experience in research immunology
15 publications in fields of virology and immunology (see attached lists)
Research Assistant, Department of Biophysics, Mellon Institute, Pittsburgh, Pa. 1964-1965
Research Assistant, The Rockefeller University, New York City 1965-1967
Research Assistant and Lecturer, Virus Chemistry Research Laboratory City University of New York 1967-1970
Senior Research Assistant, Virology Division Sloan Kettering Institute for Cancer Research, New York 1970
Research Associate, Cancer Research Unit, Department of Pathology Lenox Hill Hospital 1970 to present

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Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Edward P. Paluch	Research Associate	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED	REDACTED	REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
University College New York University	B.A. Biology	1974
Columbia University	Ph.D. Candidate	

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Human Tumor Immunology	Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Three years of experience in research immunology
4 publications in the field of immunology (see attached lists)
Research Assistant, Department of Pathology, Lenox Hill Hospital
100 East 77 Street, New York, N.Y. 1970-1971
Research Associate, Cancer Research Unit, Department of Pathology
Lenox Hill Hospital. 1972 to present

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Appendix #6

PUBLICATIONS FROM THIS LABORATORY PERTAINING TO THE TOPICS OF THIS PROPOSAL

1. Dorsett B.H., Ioachim H.L. - Common antigenic component in ovarian carcinomas: demonstration by double diffusion and immunofluorescence techniques. Immunol. Communications. 2:173-184, 1973.
2. Ioachim H.L., Dorsett B.H., Sabbath M., Andersson B. and Barber H.R.K. - Antigenic and morphologic properties of ovarian carcinoma. Gynecol. Oncology. 1:130-142, 1973.
3. Ioachim H.L., Sabbath M., Andersson M., and Barber H.R.K. - Tissue cultures of ovarian carcinomas. Lab. Invest. 31:381-390, 1974.
4. Dorsett B.H., Ioachim H.L., Stolbach L. and Barber H.R.K. - Isolation of tumor specific antibodies from effusions of ovarian carcinomas - submitted for publication.
5. Ioachim H.L., Dorsett B.H., Paluch E. and Barber H.R.K. - Electron microscopy, tissue cultures and immunology of ovarian carcinoma. Symposium on Ovarian Carcinoma, Reston, Virginia November 1974 - National Cancer Institute, - Monograph, in print.
6. Dorsett B.H., Paluch E. and Ioachim H.L. - Circulating antibodies reacting with epidermal cells in patients with squamous cell carcinoma of the lung - Feder. Proc. 33:597, 1974.
7. Ioachim H.L., Dorsett B.H., and Paluch E. - Cellular and humoral immune reactions to squamous cell carcinoma of the lung. XIth International Cancer Congress, Florence, Italy. 2:104, 1974.

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Appendix #6 (cont.)

8. Ioachim H.L., Dorsett B.H. and Sabbath M. - The immune response at the tumor site in lung carcinoma: evaluation of cellular reaction and recovery of tumor specific antibodies - submitted for publication.

9. Ioachim H.L., Keller S.E., Sabbath M. and Dorsett B.H. - Antigenic expression as a determining factor of tumor growth in Gross Virus Lymphoma - Progr. Exp. Tumor Res. 19:284-296, 1974.

10. Ioachim H.L., Keller S.E., Dorsett B.H. and Pearse A. - Induction of partial immunologic tolerance in rats and progressive loss of cellular antigenicity in Gross Virus Lymphoma - J. Exp. Med. 139:1382-1394, 1974.

11. Ioachim H.L., Keller S.E., and Dorsett B.H. - Transplantability, Immunological Unresponsiveness and Loss of Cellular Antigenicity in Gross Virus Lymphoma - in Symposium on Leukemogenesis - 3:301-310, Tokyo 1975.

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14. *First year budget:*

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s) even if no salary requested)

		% time	Amount
Harry L. Ioachim, M.D.	-Principal Investigator	30%	none
Sheldon C. Sommers, M.D.	-Consultant	10%	none
Brent H. Dorsett, B.S.	-Investigator	80%	
Edward Paluch, B.S.	-Investigator	30%	REDACTED

Technical

Caren Colbjornson	-Technician	70%	
Tove Bamberger	-Secretary	40%	REDACTED

Sub-Total for A REDACTED

B. Consumable supplies (by major categories)

Reagents (immunology)	1,500
Glassware, tissue culture media	1,500
Electron Microscopy (supplies and services)	1,500

Sub-Total for B 4,500

C. Other expenses (itemize)

Publication costs	500
Travel	500
Miscellaneous	500

Sub-Total for C 1,500

Running Total of A + B + C _____

D. Permanent equipment (itemize)

None

Sub-Total for D 0

E. Indirect costs (15% of A+B+C) E _____

Total request $30,000

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	6,600	-	-		33,000
Year 3	R	7,260	-	-		36,300

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies of Induction and Immunity of Experimental Lymphomas	National Cancer Institute CA 16997	40,000	1974-1977

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Detection of Antigens and Antibodies in Cancers of the Ovary	National Cancer Institute (planned)		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Harry L. Joachim

Signature [signature] Date 7/17/75

Telephone 212 (Area Code) 794-4420 (Number) (Extension)

Checks payable to

Lenox Hill Hospital

Mailing address for checks

100 East 77 Street

New York, New York 10021

Responsible officer of institution

Typed Name Louis F. Parker

Title Vice President

Signature [signature] Date 7/17/75

Telephone 212 (Area Code) 794-5141 (Number) (Extension)

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REFERENCES

1. Dorsett B.H. and Ioachim H.L. - Common antigenic component in ovarian carcinoma: demonstrations by double diffusion and immuno-fluorescence techniques - Immunological Communications 2:173-84, 1973.
2. Dorsett B.H., Paluch E. and Ioachim H.L. - Circulating antibodies reacting with epidermal cells in patients with squamous cell carcinoma of the lung - Feder. Proc. 33:597, 1974.
3. Ioachim H.L., Dorsett B.H. and Paluch E. - Cellular and humoral immune reactions to squamous cell carcinomas of the lung - XIth International Cancer Congress, Florence, Italy 2:104, 1974.
4. Ioachim H.L., Dorsett B., Paluch E., Barber H.R.K. - Electron microscopy, tissue cultures and immunology of ovarian carcinoma - Symposium on ovarian carcinoma - National Cancer Institute, Reston, Virginia November 1974.
5. Gold P., Freedman S.O. - Demonstration of tumor specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. - J. Exp. Med. 121, 439-462, 1965.
6. Klein G., Clifford P., Klein E. and Stjernsward J. - Search for tumour-specific immune reactions in Burkitt lymphoma patients by the membrane immunofluorescence reaction - Proc. Nat. Acad. Sci. USA 55:1628-1635, 1966.
7. Hellstrom I., Hellstrom K.E., Pierce G.E. and Yang J.P.S. - Cellular and humoral immunity to different types of human neoplasms Nature (London) 220, 1352-1354, 1968.

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8. Morton D.L., Malmgren R.A., Holmes E.C. and Ketcham A.S. - Demonstration of antibodies against human malignant melanomas by immunofluorescence - Surgery 64:233-240, 1968.

9. Hellstrom I., Hellstrom K.E., Evans C.A., Heppner G.H., Pierce G.E., Yang J.P.S. - Demonstration of cell-bound and humoral immunity against neuroblastoma cells. - Proc. Nat. Acad. Sci. USA 62:362-369, 1969.

10. Bubenik J., Perlmann P., Helmstein K., Moberger G. - Cellular and humoral immune responses to human urinary bladder carcinomas. Int. J. Cancer 5:310-319, 1970.

11. Hughes L.E. and Lytton B. - Antigenic properties of human tumours: delayed cutaneous hypersensitivity reaction. Brit. Med. J. 1:209-212, 1964.

12. Stewart T.H.M. - The presence of delayed hypersensitivity reactions in patients toward cellular extracts of their malignant tumors. - Cancer 23:1368-1369, 1969.

13. Eltringham J.R., Kaplan H.S. - Impaired delayed-hypersensitivity responses in 154 patients with untreated Hodgkin's disease - Natl. Cancer Inst. Monogr. 36:107-115, 1973.

14. Hersh E.M., Irwin W.S. - Blastogenic responses of lymphocytes from patients with untreated and treated lymphomas - Lymphology - 2:150-160, 1969.

15. Whitaker M.G., Clark C.G. - Depressed lymphocyte function in carcinoma of the breast - Br. J. Surg. 58:717-720, 1971.

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16. Catalona W.J., Sample W.F., Cretien P.B. - Lymphocyte reactivity in cancer patients: correlations with tumor histology and clinical stage - Cancer 31:65-71, 1973.

17. Bhattacharya M., Barlow J.J. - Immunologic studies of human serous cystadenocarcinoma of ovary - Cancer 31:588-595, 1973.

18. Hirshaut Y., Pei D.T., Marcove R.C., Mukherju B., Spielvogel A.R. and Essner E. - Seroepodemiology of Human Sarcoma Antigen (S_1) - N. Engl. J. Med. 291:1103-1107, 1974.

19. Ioachim H.L., Dorsett B.H. and Sabbath M. - The immune response at the tumor site in lung carcinoma: evaluation of cellular reaction and recovery of tumor specific antibodies - submitted for publication.

20. Baumstark J.S., Laffin R.J., Bardawil W.A. - A preparative method for the preparation of 7S gammaglobulin from human serum - Arch. Biochem. Biophys. 108:514-522, 1964.

21. Perper R.J., Okimoto J.T., Cochrum K.C. - A rapid method for purification of large quantities of antilymphocytic serum - Proc. Soc. Exptl. Biol. Med. 125:575-580, 1967.

22. Keller S.E., Ioachim H.L., Pearse T., and Silletti D.M. - Decreased T-lymphocyte levels in patients with breast cancer - Amer. J. Clin. Pathol. (in press).

23. Ioachim H.L., Keller S.E., Sabbath M. and Dorsett B.H. - Antigenic expression as a determining factor of tumor growth in Gross Virus Lymphoma - Progr. Exp. Tumor Res. 19:284-296, 1974.

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24. Ioachim H.L., Keller S.E., Dorsett B.H. and Pearse A. - Induction of partial immunologic tolerance in rats and progressive loss of cellular antigenicity in Gross Virus Lymphoma - J. Exp. Med. 139:1382-1394, 1974.

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26. Meltzer M.S., Leonard E.J., Rapp H.J., et.al - Tumor-specific antigen solubilized by hypertonic potassium chloride - J. Natl. Cancer Inst. 47:703-709, 1971.

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28. Ohno Y., Stahmann M.A. - Specific Agglutation of polyacrylamide derivatives of protein antigens. Immunochemistry. 9:1077-1085, 1972.

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31. Jaffe E.S., Shevach E.M., Frank M.M., Berard C.W., and Green I. - Nodular Lymphoma - evidence for origin from follicular B lymphocytes. N. Engl. J. Med. 290:814-819, 1974.

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32. Schmidt E.C., Dorsett B.H., Paluch E., and Ioachim H.L. - Membrane immunoglobulins of human lymphoma cells studied by immunoelectron microscopy. J. Cell Biol. 63:301, 1974.

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THE IMMUNE RESPONSE AT THE TUMOR SITE IN LUNG CARCINOMA: EVALUATION OF CELLULAR REACTION AND RECOVERY OF TUMOR SPECIFIC ANTIBODIES.

10035405595

Submitted for publication
July 1975.

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Cellular reaction and tumor specific antibodies in lung carcinoma.

Running title:

Immune response in lung carcinoma.

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Abstract

The local immune response to lung cancer was investigated by histologic and immunologic means. Distinctive patterns of stromal cellular reaction, characteristic for different histologic types of lung carcinoma, were recognized. The amount of cellular infiltration was higest in squamous cell carcinomas and lowest or non-existent in oat cell carcinomas. Within the various histologic categories the well differentiated tumors appeared to be accompanied by more reactive cells than the poorly differentiated and there was no relation between tumor necrosis and cellular infiltration. The plasma cells were distinctly associated with squamous cell carcinomas and their number in the stroma was proportionate to the degree of differentiation and the presence of keratin produced by the tumors.

Eluates with a high content of immunoglobulins were recovered from pleural effusions and from solid lung carcinomas by dissociation of antigen-antibody complexes. These preparations reacted positively in indirect immuno-fluorescence tests with tissue cultures and with fresh suspensions of lung carcinoma cells but not with tissue culture cells of most non pulmonary tumors or of normal adult and fetal lung. Similarly prepared fractions of non carcinomatous pleural effusions did not react with lung cancer cells.

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According to modern concepts of tumor immunology, cancer cells may express altered antigenic properties acquired as a result of neoplastic transformation. The existence of tumor-associated antigens has been clearly demonstrated in both experimental (1-8) and human (9-23) tumors.

In cancer patients the search for specific tumor antigens is based primarily on skin tests (14-15,17,24-29) and on assays of lymphocyte reactivity to tumor extracts and tumor cells (17,29-36). A sustained effort is also being made to detect circulating antigens in sera of cancer patients and several tumor-associated antigens have been described (6,9-13,18-23,37-39). However, to date, most of these efforts have not yet produced the expected results, possibly because tumor antigens in the circulating blood are diluted to infinitesimal amounts or

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locked in antigen-antibody complexes. Similarly, the lymphoid cells specifically primed to react to a particular tumor are highly diluted in the pool of circulating lymphocytes.

It appeared to us that the tumor site would be a logical place to explore the presence of tumor-reactive populations of lymphoid cells as well as to attempt the detection of tumor-associated antigens and antibodies.

To evaluate the morphology of the local cellular reaction to cancer of the lung we examined comparatively sections of 50 cases of different types and grades of lung carcinomas. The aim was not to establish clinical-pathologic correlations but to assess the presence of a cellular reaction and to estimate its intensity and its composition in regard to the various types and grades of lung carcinomas. To investigate the function of these cellular infiltrates we attempted the recovery, identification and quantitation of the immunoglobulins at the tumor site. Using solid tumors and pleural effusions, we obtained eluates with a high content of immunoglobulins showing tumor specificity. Considering that a large amount of antibodies are probably bound in antigen-antibody complexes we applied techniques designed to dissociate such complexes and to permit the recovery of active antibodies.

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Materials and Methods

A. Pathology

1. Selection of Cases

Fifty recent consecutive cases of lung carcinomas from the files of our department, were studied. All were surgical specimens, lobectomies or pneumonectomies, that were routinely processed for microscopic examination. In each case an average of 10 slides comprising Bouin-fixed, trichrome-stained (phloxine-hematoxyline-safranin) sections were examined.

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2. Evaluation of Tumor.

To characterize the tumor we considered its histologic diagnosis, degree of differentiation, amount of necrosis and capacity for invasion. The lung carcinomas examined were diagnosed as squamous, adeno, bronchiolo-alveolar, oat cell and undifferentiated carcinomas.

The degrees of differentiation were estimated on a scale from 1 to 4 as undifferentiated -1-, poorly -2-, moderately -3-, and well differentiated -4-. The amount of necrosis was similarly evaluated from 1 to 4, in relation to the total mass of tumor tissue in a microscopic field.

The capacity for tumor invasion was estimated in relation to the invasion of pulmonary tissues, blood vessels and hilar lymph nodes. Tumor invasion of local tissues appeared as rounded nodules -1-, elongated

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cords -2-, monocellular files -3- and single tumor cell invasion -4- and were considered to indicate increasing degrees of aggresiveness.

3. Estimation of Cellular Reaction

Three parameters were considered for the estimation of the cellular reaction accompanying the tumor: its amount, its composition and its distribution. The amount of cellular infiltrate was estimated on a scale from 1 to 4 by comparing in each microscopic field the surfaces occupied by tumor tissues and by cellular infiltrates, respectively. The composition of cellular reactions was marked with a P (plasma cells), L (lymphocytes) or N (neutrophils) when any of these types of cells constituted over 75 percent of the infiltrates and P.+ L (plasma cells + lymphocytes) when each of the latter were represented in about equal amounts. The distribution of the cellular infiltrate was considered in relation to the tumor cords as well as to bronchi and blood vessels. The reaction was designated PERI when the cellular infiltrate encircled only the periphery of the tumor nodule, INTER when it surrounded each tumor cord and INTRA when reactive cells penetrated between and/or in tumor cells.

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The presence, amount and distribution of fibro-collagen were also estimated on a scale from 1 to 4.

Morphologic evidence of tumor cell destruction by reactive cells was

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separately recorded. Also noted were occasional cellular infiltrates that have accumulated around bronchioles or blood vessels.

B. Immunology

1. Recovery of Immunoglobulins from Solid Tumors.

Solid tumors were finely minced and a 20% homogenate in saline prepared by twenty strokes of a tight fitting Dounce homogenizer at 4°C. The homogenate was slowly adjusted to pH 3.0 by the dropwise addition of 0.1N HCl and maintained at that pH for 30 minutes at 4°C with constant stirring. Acid insoluble precipitate was removed by centrifugation at 1500 x g for 10 minutes and the immunoglobulins were precipitated by slow addition of one-third volume of saturated NaCl. Precipitated immunoglobulins were collected by centrifugation at 1500 x g for 15 minutes and redissolved in borate buffered saline pH 8.0.

2. Recovery of Immunoglobulins from Tumor Effusions

Fluids of pleural effusions were collected under sterile conditions and clarified by centrifugation at 40,000 x g for 30 minutes at 4°C. The supernatant was precipitated by the slow addition of one-half vol. of saturated ammonium sulfate (SAS) at room temperature, with continuous stirring. After fifteen minutes, the precipitate was collected by centrifugation for 10 minutes at 5,000 x g. The pellets consisted primarily of gamma and beta globulins, as assessed by immunoelectrophoresis; they were redissolved in borate buffered saline pH 8.0 and the SAS precipitation repeated 2 times. The final pellets were redissolved in glycine-HCl buffer,

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pH 2.8 at 25°C. The pH was monitored and maintained at pH 2.8 for 15 min. with constant stirring to allow for dissociation of antigen-antibody complexes. The solution was briefly centrifuged at high speed to remove any acid insoluble material and the immunoglobulin fraction recovered by precipitation with 1/3 volume of saturated sodium chloride. The precipitated globulins were immediately recovered by centrifugation for 10 minutes at 5,000 x g at 4°C and redissolved in borate buffered saline. The purity of the globulin fraction thus obtained was assessed by immunoelectrophoresis and the quantities recovered were determined by radial immunodiffusion.

3. Immunoelectrophoresis.

Analysis of immunoglobulins was performed by electrophoresis of samples in 1% agarose in barbitol buffer ionic strength 0.075 pH 8.6 with a constant current of 30 mA for 35 minutes at 4°C. Goat anti human serum antisera (Hyland Laboratories) was added to preformed troughs and allowed to diffuse for 24 hours at room temperature.

4. Quantitation of Immunoglobulins

Antibody yields were assessed by performing radial immunodiffusion tests in high and low level Quantaplates (Kallsted Laboratories). Quantitative determination were performed for IgG, IgA, IgM and IgD on all antibody preparations.

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5. Tissue Cultures

Pleural effusion fluids collected under sterile conditions were sedimented by a 10 minute centrifugation at 200 x g then resuspended in 1-2 x 10^6 cells per ml in Eagle's minimal essential medium (MEM) supplemented with 15% fetal bovine serum and 10% human cord serum plus 1% antibiotics. The cells were grown as monolayers in Petri dishes placed in a CO_2-gassed incubator. Solid tumors were finely minced and in some cases trypsinized with 0.25 per cent trypsin in Hank's fluid for 30 minutes at 37°C. They were then resuspended in Eagle's medium and seeded as above. All cultures were subcultured by trypsinization at periodic intervals according to growth, and examined under the phase contrast microscope and on Giemsa-stained coverslips placed in the tissue culture dishes as previously described (40).

6. Direct Immunofluorescence of Tissue Sections.

Small portions of solid tumors for direct immunofluorescence were covered with OTC embedding compound (Ames Laboratories)and quicly frozen at -80°C. Cryostat sections 3 microns thick were air dried and fixed in acetone for 1 minute. Sections were covered with appropriately diluted FITC-labeled goat antihuman globulin antiserum and incubated at 37°C for thirty minutes. Sections were then washed 3 times for 5 minutes in phosphate buffered saline (PBS), and cover-slipped with 10% PBS pH 7.4 in glycerol.

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7. Indirect Immunofluorescence of Cell Suspensions and Tissue Cultures.

Slides for cytoplasmic indirect immunofluorescence assays were prepared from both solid tumors and in vitro cultured cells. Solid tumors were finely minced and the resultant single cell suspension washed twice in PBS at pH 7.2. These cells or cells directly from tissue culture were suspended at a concentration of 1×10^6 cells/ml in Eagle's MEM medium with 10% fetal bovine serum. A drop of cell suspension was placed directly on each well of 8 spot fluoroglide-sprayed slides and incubated 17 hours in a humid 5% CO_2 atmosphere at 37°C to allow cell attachment. The slides were then washed for 10 minutes in PBS and fixed in acetone for 5 minutes. Slides thus prepared were stored at -80°C until used. Indirect immunofluorescence assays were performed by placing appropriate dilutions of antibody on each spot of prepared slides and incubated in a humid chamber for 30 minutes at 37°C . The slides were then washed three times in PBS for 5 minutes and once in deionized water for one minute. After air drying, appropriately diluted F.I.T.C. labeled anti human IgG, IgA or IgM was applied to each spot. The slides were incubated as before and washed 3 times for 5 minutes in PBS, counterstained in 0.01 % Evans blue for 30 seconds and washed 5 minutes in deionized water. After drying the slides were cover slipped with 10% PBS pH 7.4 in glycerol and examined by incident fluorescent illumination.

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Results

A. Estimation of Cellular Reaction to Lung Carcinoma

Fifty lung carcinomas were examined according to various parameters and an attempt was made to establish correlations between the characteristics of the tumors and those of their stromal cellular reaction. All histologic types of lung carcinoma were represented in proportions similar to those usually encountered, (squamous cell carcinoma 21, adeno carcinoma 14, alveolar carcinoma 2, oat cell carcinoma 5, undifferentiated carcinoma 6 and metastatic adeno carcinoma 2.

The amount of tumor necrosis was inversely proportional to the degree of tumor differentiation, lowest (necrosis = 1.0) in the well differentiated alveolar carcinomas (differentiation = 4.0) and highest (necrosis = 2.8) in oat cell carcinomas (differentiation = 1.0). The invasion of the surrounding tissues was highest in oat cell carcinomas (tissue invasion = 3.8) and lowest in metastatic adeno carcinoma (tissue invasion = 1.5). The ratio of lymph nodes involved by metastatic carcinoma did not directly correlate with the histologic features being also related to the location, size and duration of tumor.

The amounts of cellular infiltration in and around lung carcinomas clearly correlated with their histologic types and degrees of differentiation. When the estimates of cellular amounts were averaged it was noted that well differentiated tumors had significantly more cellular infiltrates than poorly or undifferentiated tumors. Squamous cell carcinomas, well differentiated, (differentiation = 3.5) had the largest amounts of cellular infiltrates (reaction amount = 3.4) (fig. 1) whereas oat cell carcinomas (differentiation = 1.0)

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were almost entirely devoid of cellular infiltrates (reaction amount = 0.7) (fig.2). This contrast between the very large cellular infiltrates of squamous cell carcinomas that sometimes exceeded the amount of tumor tissue and their absence in oat cell carcinomas was striking and consistent.

The distribution of reactive cells was mostly at the periphery of tumor nodules (PERI) in the less differentiated tumors and in between (INTER) tumor cords and tumor cells in the squamous cell carcinomas. When the amount of cellular infiltration was high (reaction amount = 3 to 4) such as in squamous cell carcinomas, lymphocytes were often seen within tumor cells (INTER)(fig. 3-4).

Destruction of tumor cells, singly or in groups, surrounded by abundant agglomerates of lymphocytes and plasma cells was frequently noted in these tumors (fig.4). The appearance of single tumor cell death due to lymphocytic activity was entirely distinctive from the ischemic necrosis of tumor tissue that involved larger areas and was not accompanied by lymphoplasmacytic infiltration (fig.5). Lymphocytes within epithelial tumor cells, previously described under the name of emperipolesis (41) was often observed in areas of tumor destruction (fig.3-4). Another unusual appearance, tumor cells engulfed by tumor cells was occasionally recorded (fig.6). As in our earlier observations this was noticed almost exclusively in squamous cell carcinomas, particularly those poorly differentiated with marked anaplasia and giant tumor cells (41).

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Of all parameters used, the composition of cellular infiltrates correlated best with the histologic type of lung carcinoma. Plasma cells were present in large amounts in the squamous cell carcinomas (fig. 7) while few or none were seen in other histologic types. The well differentiated squamous cell carcinomas that included the formation of keratin, exhibited the greatest numbers of plasma cells. Not unusually in such tumors, plasma cells were the only constituent of stromal cellular reactions, occasionally forming huge sheets of cells that on occasion exceeded the size of tumor cords (fig. 1, 7). It appeared that a direct correlation existed between the presence of keratin or keratin precursors in squamous cell carcinomas and the amount of plasma cells in the surrounding stroma. In the adeno carcinomas, plasma cells were present mostly in areas of squamous metaplasia. In lymph nodes with tumor metastases, numerous plasma cells were present in contact with the tumor, particularly when this was squamous cell carcinoma. On occasion, huge areas of lymph nodes invaded by metastatic squamous cell carcinoma were transformed in sheets of plasma cells.

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Fibrosis was noted in almost all lung carcinomas however its degree and distribution were variable. In general, the amount of fibro-collagenous tissue around (PERI) and within (INTER) the tumor nodules was indirectly proportional to the amount of cellular infiltrates. Tumors with abundant cellular reaction showed little proliferation of collagen fibers (fig. 1, 7) whereas tumors with few or no cellular infiltrates contained broad, dense bands of collagen (fig. 2). Even within the same tumor there were areas of fibrosis with poor cellularity, in contrast to areas of abundant cellular reaction that did not include a sizable fibrous component. Metastatic carcinomas to the lung showed patterns of

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cellular infiltrates similar in size and composition to those of primary carcinomas of same histologic type.

B. Isolation of tumor specific antibodies

Cryostat sections of lung carcinomas were reacted with FITC-labeled antihuman IgG, IgM and IgA antisera and examined under the fluorescence microscope. It was observed that the plasma cells and lymphocytes of the stromal reaction exhibited strong intracytoplasmic fluorescence to all three immunoglobulins although IgG-positive cells were predominant (fig. 8). To investigate the nature and specificity of such immunoglobulins, eluates of lung carcinomas as well as fluids of pleural effusions were processed.

When solid lung tumors were eluted with acid buffer and the immunoglobulins recovered by salt precipitaton, an average of 2mgm IgG per gram of tumor tissue was obtained. IgA and IgM in substantially lower amounts were also recovered. Indirect immunofluorescence tests performed with these eluates and monospecific conjugates showed apparently specific binding of IgG to lung carcinoma cells grown in tissue culture. Three of four eluates of solid lung carcinomas (table 2) adjusted to a concentration of 2 mgm/ml and tested at a 1:10 dilution, exhibited granular cytoplasmic fluorescence with tissue culture cells of a pulmonary adenocarcinoma (fig. 9). In addition, two of these preparations gave similar immunofluorescence patterns with cultures of squamous cell carcinomas of the skin and larynx. All four eluates were negative when tested against normal fetal and adult lung cell suspensions as well as against tissue culture cells of non pulmonary carcinomas (table 2).

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Pleural effusions accompanying lung carcinomas were also assayed for the presence of tumor specific antibodies. Freshly tapped fluids assayed in indirect immunofluorescence against lung carcinoma cells, did not show any reactivity. However, when the antigen-antibody complexes were dissociated, the IgG fraction recovered by salt precipitation exhibited substantial tumor reactive antibody activity. In immunoelectrophoresis this acid-treated immunoglobulin fraction was found to consist almost entirely of IgG with very little contamination of other serum proteins (fig.10).

IgG fractions prepared from two lung carcinoma effusions were tested in the indirect immunofluorescence assay and found to produce tumor cell staining patterns similar to those obtained with acid eluates of solid lung tumors (fig.11). Both of these IgG fractions prepared from 500 ml of effusion fluids consisted of about 50 ml of IgG at 4.0 mg/ml and gave positive tumor cytoplasmic immunofluorescence at a dilution of 1:64. The fractions reacted positively with tissue culture cells of both squamous cell and adeno carcinoma of the lung and failed to react with cell suspensions of normal human tissues or of tissue culture cells of non pulmonary carcinomas (table 3). Antibody fractions similarly prepared from pleural effusions of patients with lymphosarcomas, ovarian and breast carcinomas, oat cell pulmonary carcinoma and renal failure failed to give positive immunofluorescence staining with lung carcinoma tissue culture cells. In addition to the intracytoplasmic fluorescence produced by the antibody preparations of cancer effusions, nucleolar staining was also observed in all carcinoma cells reacted with these antibody fractions. This may indicate the presence of tumor-associated nucleolar antigens detected by our immunoglobulin extracts. Similar nucleolar antigens reacting with autologous and homologous sera in indirect immunofluorescence were recently described in melanoma patients (42).

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Discussion

The infiltration of cancertissue by reactive cells has long been noticed by pathologists who have regarded it as an indication of host resistance to tumor invasion. To express this idea, Russel (1908) coined the term "stroma reaction" (43) while Murphy (1921) advanced the view that "immunity to cancer, whether natural or induced, is attended by lymphoid stimulation" (44-45). Ewing (1940) similarly regarded the inflammatory reaction accompanying tumors as a defensive process (46) and a number of subsequent histologic studies attempted to demonstrate a positive correlation between stromal reaction and prognosis (47-54). However, counter arguments were furnished by equally prestigious authors (Woglom (1929) (55) Willis (1953) (56)) as well as by several histologic studies (57-59), to show that the case for the tumor-antagonizing activity of lymphocytes was not yet proved (60).

It is notable that the concept of stromal reaction, which preceded by half a century the modern theories of cellular immunity to cancer, is not used for practical purposes today. In fact, the stromal reaction of tumors is hardly ever mentioned in pathology reports and there are no accepted criteria for its evaluation. To assess the significance of stromal reaction, standardized data regarding a large spectrum of tumors are obviously needed. On the other hand, the modern studies of cancer immunity that use a diversified and complex methodology to investigate the function of lymphocytes and antibodies seem to ignore the cellular reaction at the tumor site and are almost exclusively concerned with the cell populations of the general circulation.

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Considering that the host's response to a tumor must be maximal at the site of the tumor, we have attempted the investigation of the local cellular and humoral reactions. To estimate the cellular infiltration at the tumor site, we have used a set of histologic criteria designed to permit quantification

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and comparison. These criteria are certainly imperfect due to the many variables inherent to the study of tissue sections. However, they can provide, if consistently applied, a fairly accurate picture of the stromal reaction of various tumors. Thus it was possible to recognize patterns of cellular reaction that were characteristic for different histologic types of lung carcinoma and that seem to be applicable to tumors of other tissues as well.

The degree of cellular infiltration was highest in squamous cell carcinomas and lowest or nonexistent in oat cell carcinomas (table 1). Within the various histologic categories the well differentiated carcinomas appeared to be accompanied by more reactive cells than the poorly differentiated.

The plasma cells were distinctively associated with squamous cell carcinomas and their number in the stroma of such tumors was proportionate to the degree of differentiation and the presence of keratin produced by the tumor. In a morphologic study of the stromal reaction of various malignant tumors, now in preparation, we have found that the selective association of plasma cells with squamous cell carcinoma occurs not only in lung tumors but in squamous cell carcinomas of all other organs so far examined (tongue, esophagus, cervix, endometrium, anus and skin).

In searching for a biologic meaning to these correlations, it appears that lymphocytes, and plasma cells accumulate in and around tumor tissues in response to specific antigenic stimulation. This is not a nonspecific reaction to infected or necrotic tissues, as it has been frequently assumed because 1. the cells responding to infected or necrotic tissues are invariably polymorphonuclears and not lymphocytes and plasma cells and 2. the present study

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clearly shows that the carcinomas with the greatest amount of necrosis (oat cell, undifferentiated carcinoma) have the least amount of cellular infiltration (table 1). The correlation between degree of tumor differentiation and amount of cellular infiltrate further strengthens the assumption that the reactive cells are specifically attracted to the tumor site by tumor-associated antigens. Since undifferentiated carcinomas are not accompanied by cellular infiltrates, it may be assumed that such tumor cells are less antigenic. It is possible that, during the progression of tumors, as a result of clonal selection, new cancer cells emerge that are poorly differentiated and exhibit little or no antigenic expression. Such cells are therefore more likely to escape immunologic recognition and annihilation and to give rise to cancers of increased resistance and aggressivity.

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Lymphocytes and plasma cells may respond to a large variety of tumor-associated antigens. In the case of squamous cell carcinomas the presence of large numbers of plasma cells seems to be attributable to the capacity of these tumors to produce keratin. We have documented earlier the association of plasma cells with keratinized squamous epithelia in several other contitions, not all neoplastic. We have also shown that sera of patients with pulmonary squamous cell carcinomas reacted specifically against cutaneous squamous epithelium in indirect immunofluorescence and with a soluble antigen extracted from such epithelium, in complement fixation tests (20,22).

In the present study just as plasma cells were associated with squamous cell carcinomas, deposition of dense acellular collagen appeared to be associated with oat cell carcinomas. There are tumors of other organs, infiltrating duct

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carcinoma of the breast being a notable example, that appear to induce a fibro-collagenous reaction rather than a cellular infiltrate.. This might represent another pattern of reaction to tumor growth, characteristic for tumor cells of a particular antigenic expression.

At the present time we know little about the nature and variety of human tumor-associated antigens. However, as these antigens will become better defined in the future we may find that various patterns of stromal reactions appropriately match the categories of tumors and the types of antigens.

The large numbers of plasma cells and lymphocytes present in the tumor stroma and their apparently selective association with different tumor types suggested that tumor specific antibodies might be released by these cells and that the tumor site would be the logical place to attempt their recovery. This assumption was supported by several recent studies showing that low pH eluates of experimental (61) as well as of human (38, 62-65) tumors contain immunoglobulins and that these tumor-bound antibodies may block lymphocyte-mediated cytotoxicity in vitro (61, 63).

In the present study, when cryostat sections of lung carcinomas were stained with antihuman immunoglobulins in direct immunofluorescence tests the cellular infiltrates of plasma cells and lymphocytes, reacted positively for all three immunoglobulins IgG, IgM and IgA. The eluates recovered from solid lung carcinomas reacted positively in indirect immunofluorescence with tissue-cultured lung adeno and squamous carcinoma cells but not with cell suspensions of normal lung or tissue-cultured cells of 15 nonpulmonary tumors, with the exception of two squamous cell carcinomas of skin and larynx.

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The results obtained with eluates of pleural effusions were even more encouraging. IgG fractions prepared from two lung carcinoma effusions produced strong cytoplasmic staining in indirect immunofluorescence with all four lung carcinoma tissue-cultured cells tested and with none of 7 cultures of nonpulmonary tumors and of 3 normal fetal and adult lungs. Eluates of 2 non carcinomatous pleural effusions did not react with lung cancer cells in indirect immunofluorescence.

Although so far only a limited number of cases have been assayed, the results were consistent in indicating that tumor specific antibodies were present at the tumor site in amounts significantly larger than in the general circulation. It also appeared that these immunoglobulins were largely the product of the plasma cells and lymphocytes accumulated in the tumor stroma. It is presently not clear whether antibodies produced against tumor-associated antigens are beneficial to the host in the fight to eradicate the tumor. Some studies indicate that such antibodies may in fact abrogate the in vitro cytotoxic activity of lymphocytes by blocking the receptor sites on tumor cells (61, 63, 66) thus enhancing the progression of the tumors (61, 63). However, final conclusions on this matter are not yet at hand since in vitro-in vivo comparisons were frequently inconclusive.

In our work we did not attempt to correlate directly the type or amount of stromal cellular reaction with clinical events such as the recurrence of tumors or survival of patients because it appeared that more than one factor must be operative in these complex events. However, having shown that the degree of cellular reactions correlates positively with the histologic differentiation of lung carcinomas, this study implicitly indicates that local cellular reactions

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correlate with better prognoses, since patients with squamous cell carcinomas have longer survivals than patients with oat cell carcinomas, and well differentiated tumors appear to behave less malignantly than poorly differentiated tumors of the same histologic type. Whether these correlations indicate causal or merely coincidental relationships cannot be presently determined. Perhaps at a stage as late in the evolution of a tumor as its surgical removal, the stromal reaction may no longer reflect the capacity of the host to contain the growth of his tumor. Conversely, a correlation between the stromal reaction and the outcome of a malignant tumor might be established if the tumor progression were considered from the time of its inception, assuming that one knew how to determine the time of that event.

While these considerations might still be considered theoretical at the present time, the isolation of immunoglobulins from tumor tissues has important and far-reaching practical purposes. The recovery of tumor-associated immunoglobulins from antigen-antibody complexes can provide autologous immunoglobulins that do not require exhaustive absorption preparation, in amounts much greater than those obtainable from serum. Such immunoglobulins represent pure tumor-antibodies that can be used as highly specific reagents to detect the presence of tumor antigens. Our results with immunoglobulins recovered from solid tumors and pleural effusions in lung carcinoma (20, 22) and from peritoneal effusions in carcinoma of the ovary (18, 21, 23) indicate that the recovery of these immunoglobulins is feasible.

Obtaining pure, autologous, tumor-specific antibodies appears a most desirable aim for their potential use in cancer immunodiagnosis and immunotherapy.

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Legend of Figures

Fig. 1 Squamous cell carcinoma, well differentiated showing solid tumor cords surrounded (PERI), divided (INTER) and infiltrated (INTRA) by large amounts of plasma cells and lymphocytes. (Tumor = T. Stroma = S). x 100

Fig. 2 Oat cell carcinoma. Tumor cords surrounded by fibrous stroma lacking any cellular infiltrates. (Tumor = T. Stroma = S). x 100

Fig. 3 Squamous cell carcinoma, moderately differentiated. Island of tumor tissue (T) surrounded and heavily infiltrated by lymphocytes (L) that penetrate the tumor cells, occasionally . (Emperipolesis = E). x 100

Fig. 4 Squamous carcinoma cells surrounded and infiltrated by plasma cells and lymphocytes (Emperipolesis = E). Alterations of tumor cells indicate degeneration and death; degenerative changes = D). x 250

Fig. 5 Undifferentiated carcinoma (T) with extensive area of necrosis (N), entirely devoid of cellular infiltrates. x 250

Fig. 6 Squamous cell carcinoma, well differentiated, showing frequent cytophagocytosis (tumor cells engulfed by tumor cells).
Tumor cell partly (1) or entirely (2) degenerated within phagocytic vacuoles of tumor cells. x 250

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Fig. 7 Well differentiated squamous cell carcinoma (T) with abundant stromal cellular reaction composed almost entirely of plasma cells (P); (emperipolesis = E). x 250

Fig. 8 Cryostat section of lung squamous cell carcinoma stained with FITC-labeled goat antihuman IgG in direct immunofluorescence, showing rich infiltrate of antibody-secreting cells. x 400

Fig. 9 Tissue culture of lung adenocarcinoma reacted with IgG eluted from a different lung adenocarcinoma (solid tumor), in indirect immunofluorescence, showing positive cytoplasmic and nucleolar staining. x 250

Fig.10 Immunoelectrophoresis plate comparing patterns of untreated pleural effusions (1 and 3) with patterns of immunoglobulins recovered by acid dissociation and salt pricipitation (2 and 4). Troughs (A) contain goat anti human serum antisera. Patterns 2 and 4 indicate that the recovered immunoglobulin fractions consist almost entirely of IgG.

Fig.11 Tissue culture of lung squamous cell carcinoma reacted with IgG fraction recovered from pleural effusion of lung adenocarcinoma in indirect immunofluorescence, showing positive cytoplasmic and nucleolar staining. x 400

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Table 1. Stromal Cellular Reaction in Lung Carcinomas

TUMOR				INVASION			REACTION							
								Composition			Distribution			
Type[1]	Cases[2]	Differ.[3]	Necrosis	Tissues	Vessels	L.N.[4]	Amount	P[5]	L[6]	P + L[7]	Peri	Inter	Intra	Fibrosi
Squamous Carcinoma	16	3.5-well	1.3	2.4	0.6	6	3.4	9	3	4	3	3	10	3.7
	5	2.0-poor	1.4	2.6	0.8	6	2.8	1	3	1	2	1	2	2.2
Adeno Carcinoma	9	3.4-well	1.4	2.3	1.0	3	2.0	0	7	2	5	3	1	1.9
	5	1.7-poor	2.6	2.1	0.5	2	1.7	0	3	2	3	2	0	2.1
Alveolar Carcinoma	2	4.0-well	1.0	2.5	2.0	0	3.4	0	0	2	1	1	0	1.0
Undiffer. Carcinoma	6	1.0-un[8]	2.7	3.3	1.0	6	2.1	1	2	3	3	2	1	2.1
Oat Cell Carcinoma	5	1.0-un[8]	2.8	3.8	1.8	8	0.7	0	3	0	3	0	0	2.2
Metast.[9] Adeno Ca.	2	3.0-well	1.0	1.5	1.0	0	2.5	0	1	1	2	0	0	2.0

1 = Histologic Type
2 = Number of Cases
3 = Degree of Differentiation
4 = Lymph Nodes
5 = Plasma Cells
6 = Lymphocytes
7 = Plasma Cells + Lymphocytes
8 = Undifferentiated
9 = Metastatic Adeno Carcinoma (1 breast; 1 colon)

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Table 1. Stromal Cellular Reaction in Lung Carcinomas

TUMOR				INVASION			REACTION							
								Composition			Distribution			
Type[1]	Cases[2]	Differ.[3]	Necrosis	Tissues	Vessels	L.N.[4]	Amount	P[5]	L[6]	P + L[7]	Peri	Inter	Intra	Fibrosis
Squamous Carcinoma	16	3.5-well	1.3	2.4	0.6	6	3.4	9	3	4	3	3	10	3.7
	5	2.0-poor	1.4	2.6	0.8	6	2.8	1	3	1	2	1	2	2.2
Adeno Carcinoma	9	3.4-well	1.4	2.3	1.0	3	2.0	0	7	2	5	3	1	1.9
	5	1.7-poor	2.6	2.1	0.5	2	1.7	0	3	2	3	2	0	2.1
Alveolar Carcinoma	2	4.0-well	1.0	2.5	2.0	0	3.4	0	0	2	1	1	0	1.0
Undiffer.[8] Carcinoma	6	1.0-un[8]	2.7	3.3	1.0	6	2.1	1	2	3	3	2	1	2.1
Oat Cell Carcinoma	5	1.0-un[8]	2.8	3.8	1.8	8	0.7	0	3	0	3	0	0	2.2
Metast.[9] Adeno Ca.	2	3.0-well	1.0	1.5	1.0	0	2.5	0	1	1	2	0	0	2.0

1 = Histologic Type
2 = Number of Cases
3 = Degree of Differentiation
4 = Lymph Nodes
5 = Plasma Cells
6 = Lymphocytes
7 = Plasma Cells + Lymphocytes
8 = Undifferentiated
9 = Metastatic Adeno Carcinoma (1 breast; 1 colon)

Figures in columns: Differ., Necrosis, Tissues, Vessels, Amount, Fibrosis represent averages of estimates on a scale from 1 to 4.

Figures in column: L.N. represent quotient of total uninvolved lymph nodes divided by total of involved lymph nodes.

Figures in columns: Composition and Distribution represent number of cases.

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Table 3 Immunoglobulins of Lung Carcinoma Effusions In Indirect Immunofluorescence Tests.

Cells Tested	Effusions			
	Lung adeno Ca 1	Lung adeno Ca 2	Renal Failure	Hodgkin's Disease
Lung squamous Ca (E_{14})	+	+	-	-
Lung adeno Ca ($HLuT_{29}$)	+	+	-	-
Lung adeno Ca ($HLuT_{30}$)	+	+	-	-
Lung adeno Ca ($SKLu_1$)	+	ND	ND	ND
Breast infiltrating Ca (HBr_5)	-	-	-	-
Breast infiltrating Ca (HBr_{28})	-	-	-	-
Breast infiltrating Ca ($SKBr_3$)	-	-	-	-
Cervix squamous Ca (Me 180)	-	ND	ND	ND
Ovarian Ca (OT_{98})	-	-	-	-
Ovarian Ca (OT_{101})	-	-	-	-
Melanoma (HMT_1)	-	-	-	-
Normal fetal lung (NHLu)	-	-	-	-
Normal fetal lung (NHLu)	-	ND	ND	ND
Normal adult lung (WI_{38})	-	ND	ND	ND

Ca = Carcinoma

ND = not done

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Table 2 Immunoglobulins of Solid Lung Carcinoma Eluates In Indirect Immunofluorescence Tests.

Cells Tested	#	Lung Carcinoma Eluates El 1	El 2	El 3	El 4
Lung adeno Ca	- 2	2/2	0/2	2/2	2/2
Breast infiltrating Ca	- 2	0/2	0/2	0/2	0/2
Colon adeno Ca	- 3	0/3	0/3	0/3	0/3
Cervix squamous Ca	- 4	0/4	0/4	0/4	0/4
Bladder urothelial Ca	- 1	0/1	0/1	0/1	0/1
Kidney clear cell Ca	- 1	0/1	0/1	0/1	0/1
Endometrial adeno Ca	- 1	0/1	0/1	0/1	0/1
Vaginal adeno Ca	- 1	0/1	0/1	0/1	0/1
Skin squamous Ca	- 1	1/1	0/1	1/1	0/1
Larynx squamous Ca	- 1	1/1	0/1	1/1	0/1
Hepatoma	- 1	0/1	0/1	0/1	0/1
Melanoma	- 1	0/1	0/1	0/1	0/1
Fetal normal lung	- 1	0/1	0/1	0/1	0/1
Adult normal lung	- 1	0/1	0/1	0/1	0/1

Ca = Carcinoma

= Number of cases

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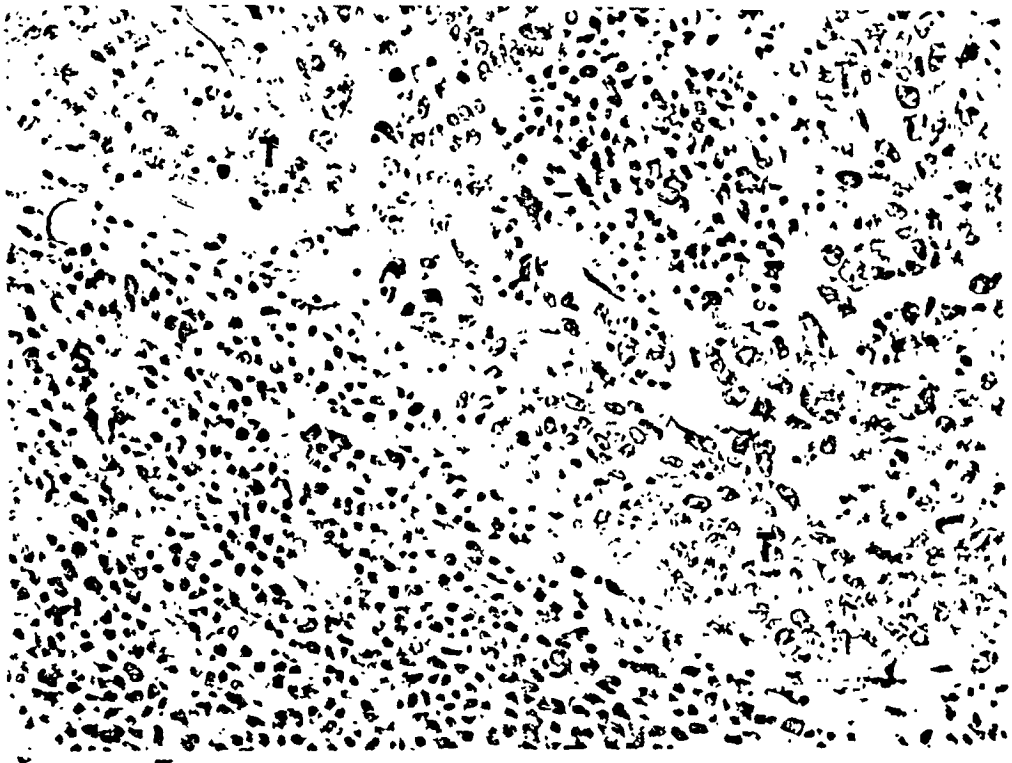

Fig 1

Fig 2

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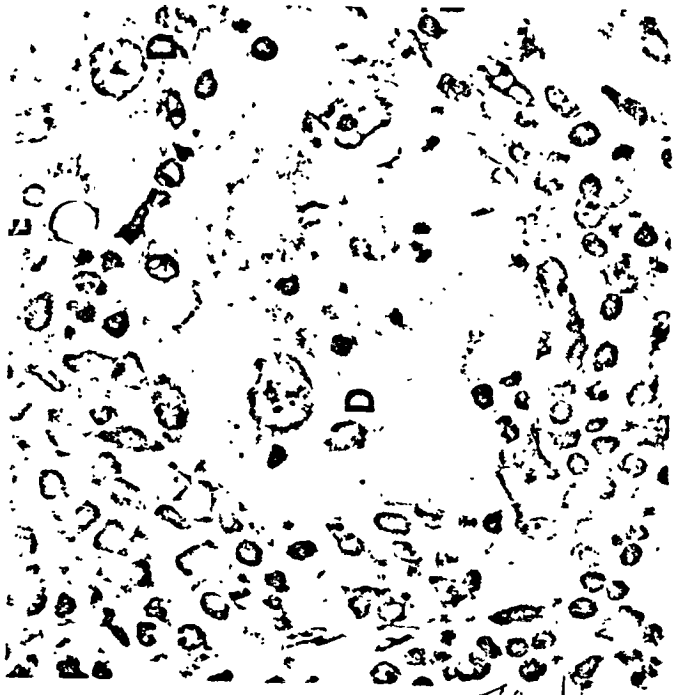

Fig 4

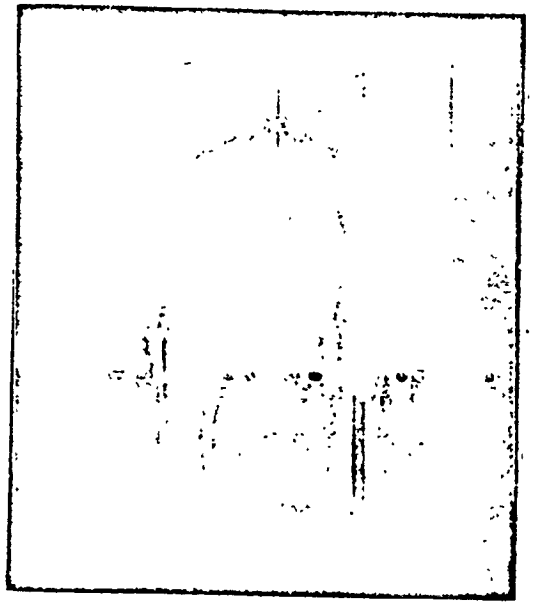

Fig 10

Fig 3

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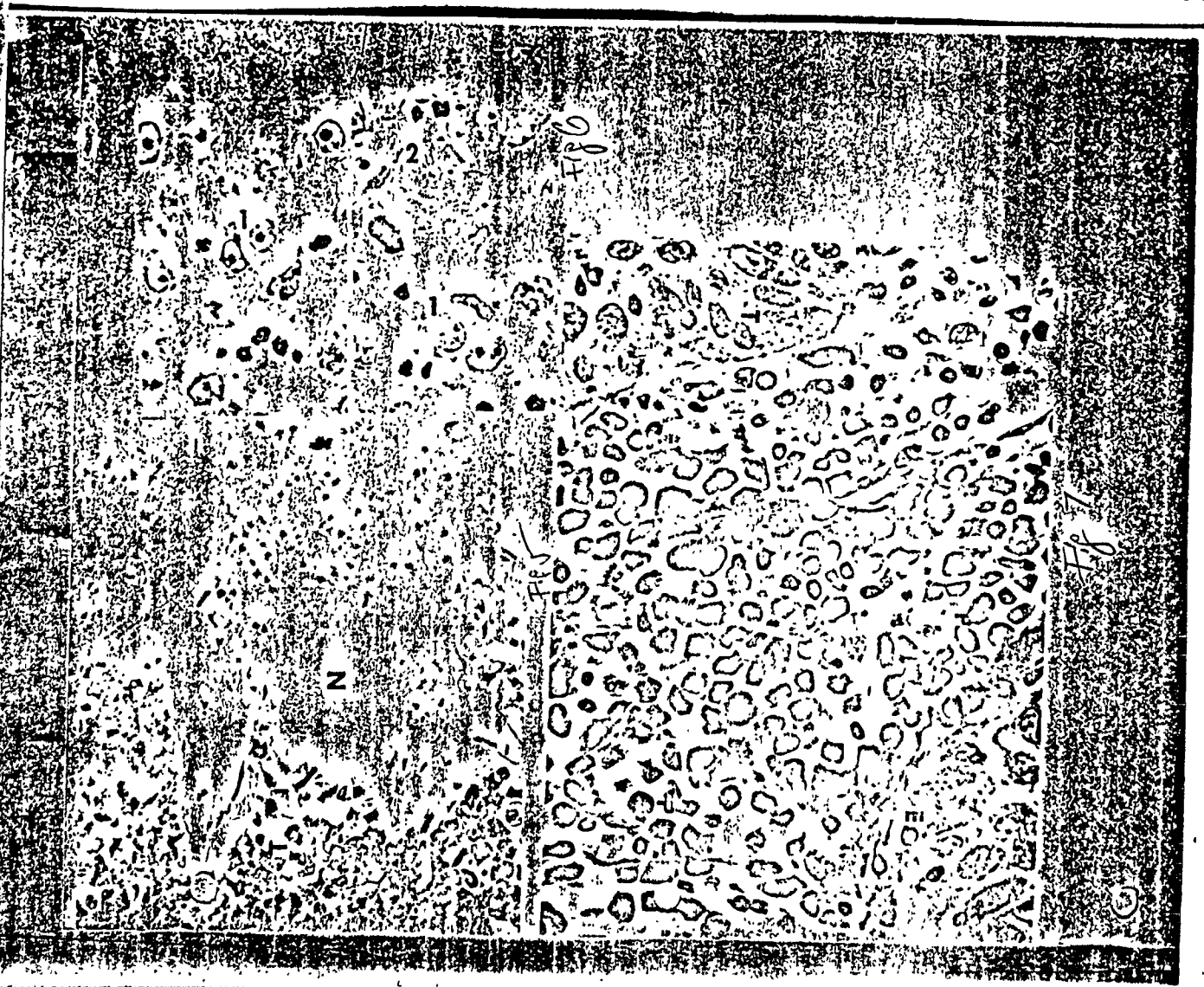

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Fig 11

Fig 9

Fig 8

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 17, 1975

Grant application No. 573D

CANCER

To: The committee comprising Drs. Gardner, Jacobson, Meier and Sommers

Subject: Clayton G. Loosli, M.D.,Ph.D.,Univ. of Souther Calif., Los Angeles
Continuation application No. 573D
"The Effects of Fresh Cigarette Smoke Inhalation on the Respiratory Tract of Mice"

History

This productive and distinguished investigator has been supported by CTR for the past nine years. In October 1974 the SAB approved a 'terminal' award (Grant No. 573C) of $60,000 for the period October 1, 1974 through September 30, 1975.

Additional funds are requested since the animals under investigation did not exhibit the expected morbidity and mortality, and, therefore, the study has not approached termination as planned. Continuation will ensure that the optimal end-points resulting from cigarette smoke exposure can be obtained: the investigation appears to be entering the critical phase of life-time, accumulative, cigarette-smoke inhalation effects.

Request

Application No. 573D requests $49,274 for a nine month continuation in order to complete the study.

Documents submitted (attached)

1. Application (7 pages - not including face sheet).
2. Letter from Dr. Loosli to Dr. Lisanti dated July 7, 1975 (3 pages).
3. Memo from Dr. Gardner dated July 15, 1975 (2 pages)
4. Two reprints.

Comment

The photographs mentioned in the Loosli letter of July 7, 1975 will be forwarded on request.

D.S.

DS:wg
Atts.

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THE COUNCIL FOR TOBACCO RESEARCH - USA

110 East 59th Street

New York, New York 10022

STATUS PROGRESS REPORT VII

AUG. 1, 1974 to JULY 1, 1975

Title: The Effects of Fresh Cigarette Smoke Inhalation on the Respiratory Tract of Mice

Investigator: Clayton G. Loosli, M.D., Ph.D.
Hastings Professor of Medicine and Pathology
University of Southern California School of Medicine, 2025 Zonal Avenue, Hoffman Research Building, Room 915, Los Angeles, Calif. 90033

Date: July 1, 1975

Confidential: To Staff and Scientific Advisory Board Only

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INTRODUCTION

The following is a brief summary of the status of our current studies being supported by the Council for Tobacco Research USA. Status progress report VI summarized studies up to August 1st, 1974. Studies which have been continued this past year will be summarized briefly.

Sendai Virus Infections: As you know, an extensive respiratory disease outbreak occurred in mice being subjected to cigarette smoke inhalation the summer and early fall of 1972. The etiological agent was identified as the Sendai virus. The surviving animals were continued to be exposed to cigarette smoke until the summer of 1973, when the animals were sacrificed, some having been exposed to cigarette smoke inhalation for nine months. The Air Pollution Building was then fumigated and new groups of animals were introduced in the late fall of 1973.

When the Sendai virus was isolated, it seemed important to set up experimental studies in order to compare the pathology of the spontaneous disease with that resulting from airborne exposure of the virus under experimental conditions. Several experiments were carried out. The pulmonary lesions were shown to be identical with those seen in the lungs of animals ill with the spontaneous illness. C57Bl/6J, C57L/J, SWR/J, and CD-1 strains of mice were equally susceptible to aerosolized Sendai virus. The lesions resemble very much those resulting from aerosol exposure of mice to the PR8-A influenza virus. Serological studies showed no cross reactions between the two myxoviruses.

Because of the tremendous cost, in time, animals, etc. of the spontaneous Sendai outbreak, it seemed important to see if subcutaneous vaccination with formalinized allantoic fluid culture of Sendai virus would protect against the experimental disease. Inactivated Sendai vaccine, given subcutaneously with and without Freund's adjuvant, to C57Bl/6J, C57L/J, SWR/J and CD-1 mice produced essentially complete protection against aerosols of virus containing several lethal airborne doses - prevention being against illness, death, and residual pulmonary lesions. One sub C dose of 0.1 to 0.15ml has been shown to be effective in preventing illness, death, and residual pulmonary lesions for 5 months. The data on the natural outbreak, experimental airborne infection, and protective effects of vaccination against Sendai virus infections, is being prepared for publication.

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These studies support those recently reported by Fukumi and Takeuchi in Japan (Fukumi, H. and Takeuchi, Y: Vaccination against parainfluenza I virus (Typus muris) infection in order to eradicate the virus in colonies of laboratory animals. International Symposium on Immunity to Infections of the Respiratory System in Man and Animals. London, 1974. Developments in Biological Standardization, Vol. 28, pp. 477-481, Karger, Basel 1975. In this article, the seriousness of the problem in keeping SPF mice and guinea pigs free of Sendai virus infections is emphasized. They conclude that vaccination against the virus is efficient and the duration of immunity satisfactory.

Cigarette Smoke Inhalation Studies: This study began in late December, 1973 and January, 1974. At the time of onset of smoke exposure, the mice were 8 to 10 weeks old. Three strains of mice, C57Bl/6J, C57L/J, and SWR/J, all Sendai free, have been exposed to fresh cigarette smoke. Smaller groups of exposed and control (sham and shelf) mice have been sacrificed at 3,6,9, and 12 months after onset. Accurate records of the number of cigarettes to which the mice are exposed are being kept. Mice exposed to the smoke of one cigarette daily show little change, after one year, in lung structure, while those exposed to smoke from four cigarettes daily show epithelial hyperplasia, epithelial alveolarization, alveolar macrophage collections and chronic pneumonitis. No bronchial metaplasia or alveolar adenomas have been observed up to 12 months of smoke exposure. The C57Bl/6J show the least bronchial hyperplasia while the SWR/J show the greatest.

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TABLE I

Cigarette Smoke Exposure Inhalation Schedule

Daily Exposure of Cigarettes Through 6-27-75

Mouse Strain	Age in Mo.	Duration in Mo.	4x Reg Diet	4x NoA Diet*	1x Reg Diet	Controls Sham Reg D	Controls Shelf Reg D
C57Bl/6J	22	20	56(1383)**	50(1350)	38(499)	25	28
C57L/J	22	20	46(1283)	48(1250)	50(373)	52	25
	TOTAL:		102	98	88	77	53
SWR/J Sacrificed 12th Through 17th, 1975							
SWR/J	21	19	18(1252)	28(1121)	38(328)	22	29

* Placed on NoA diet 2-10-75
** Total number of cigarettes per mouse

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C57Bl/6J and C57L/J Mice: It is noted in Table I that mice in these two groups, as of the end of June, 1975, will be 22 months old and will have been exposed to smoke from 1A1 cigarettes for approximately 20 months. As of February 10th, 1975, half of the two groups were placed on a vitamin A deficient diet (NoA). As of now, 98 mice, exposed to smoke from 4 cigarettes daily, have been on a NoA diet for approximately 4½ months. As the mice in the above groups are tolerating the smoke exposure well, it is thought extremely important to continue to expose them for another four months or until they are at least 26 months old. Combining both NoA and regular diet groups, two hundred mice will then be exposed to cigarette smoke four times a day for approximately 24 months. As far as I know, now, there will be no other groups of mice in America which have been exposed to cigarette smoke as consistently and as long.

SWR/J Mice: Because the SWR/J smoke exposed mice are smaller in number (see Table I), they were sacrificed over a period of a week, from June 11th through the 17th. At the time of sacrifice, the mice were 21 months old and had been exposed to cigarette smoke for 19 months. As in the past, the lungs will be examined by light and electron microscopic, histochemical and biochemical (surfactant) procedures. Also, the livers of the smoke exposed groups on NoA and regular diets and of control animals will be titrated for vitamin A . The tissue specimens from the SWR/J mice will be processed immediately for examination.

Cigarette Smoke Inhalation Employing NoA and RD C57Bl Mice Followed by Influenza Virus Infection.

Mice 10 Months Old: One hundred-sixty (160) male C57Bl mice born October, 1974, were separated into two groups February 10th, 1975 and placed respectively on NoA and regular diets. An additional sixty mice of the same strain serve as controls. As of July 10th, these mice will be 9 months old and 80 will have been on a NoA diet for 5 months.

On July 14th these will be exposed to 1A1 cigarettes daily. The exposure will be gradually increased to, if possible, 6 cigarettes daily. The cigarette exposed mice, along with the sham and controls, will be housed in the Air Pollution Building. It is hoped that these mice can be exposed as per above schedule for 6 months or until December 30th, 1975. At this time, they will be subjected to sub-lethal clouds of influenza virus.

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Smaller groups will then be sacrificed at increasing intervals of time. Comparative studies will then be made concerning the morphological and possibly the cancerous nature of the post-influenzal lesions as shown by light and electron microscopic, histochemical and biochemical procedures and subcutaneous transplantation of the post-influenza pulmonary nodules.

Mice 4 Months Old: A companion experiment to the above will be carried out using younger C57Bl/6J mice known to be vitamin A deficient as a result of having been on a vitamin A deficient diet since birth. Two hundred forty (240) mice will be employed, 120 on a regular diet and 120 on a NoA diet, plus 60 controls. After six months, they will be subjected to sublethal clouds of influenza virus, sacrificed and studied as above.

Influenza Virus Infections in C57Bl/6J Vitamin A Deficient (NoA) and Regular Diet (RD) Mice: Previous progress reports and two publications show that vitamin A deficient mice (CD-1 strain) show extensive hyalinization and keratinization of the regenerating bronchial membranes and post-influenzal epithelial nodules. This year, as has been stated, large numbers of vitamin A deficient C57Bl/6J mice have been raised by placing the pregnant females on a vitamin A deficient diet from 2 to 4 days before term and keeping the mother and offspring on the vitamin A deficient diet thereafter. After weaning, the mother is placed on a regular diet and after 6 weeks is mated again.

An experiment has just been completed and another one is in progress in which C57Bl/6J mice (NoA and RD) have been exposed to sublethal aerosols of mouse adapted influenza A viruses. As was observed in the lungs of CD-1 NoA mice, spectacular squamous metaplasia, characterized by extensive hyalinization and keratinization of the regenerating bronchial membranes and post-influenzal pulmonary nodules were seen. In the 18 and 25 day lesions, the lesions appear to be expanding (compressing surrounding lung tissue) and active. These resemble remarkably the illustrations of Nettesheim et al (J. Nat. Cancer Inst. 47: 697-701, 1971). The second experiment is similar to the first except that it will be carried on longer so that lesions from 40 to 80 days after onset can be studied. Some of the vitamin A deficient mice will be placed on a regular diet 30 days post infection to see if the squamous metaplasia and keratinization are reversed. Squamous cell post-influenzal nodules from some mice also will be transplanted subcutaneously to the same strain of NoA and RD C57Bl/6J mice. This study will continue through November, 1975.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Influenza PR8-A Virus Infection in NoA and RD C57Bl/6J Mice Previously Inoculated Intratracheally with Methylcholanthrene (MCA) and Benzo(a)pyrene (BaP): C57Bl/6J male and female mice raised on regular (RD) and vitamin A deficient diets (NoA) will be inoculated intratracheally after the method of Nettesheim et al (J. Nat. Cancer Inst. 47: 697-701, 1971).

BaP (Benzo(a)pyrene): The carcinogens will be suspended in sterile saline containing 0.20 percent gelatin. The animals will be anesthetized with IP pentobarbital and 0.3mg of BaP at six weekly intervals. Four weeks after the last injection, smaller groups will be subjected to sublethal clouds of influenza virus, along with NoA and RD mice not injected with carcinogens. This experiment is designed to compare the pathogenesis and pathology of the post-influenzal lesions in the different mouse groups and to determine if the influenza virus might act as a cocarcinogen.

This study will begin July 21st with at least 250 C57Bl/6J mice of the same age, half of which will be vitamin A deficient.

MCA (methylcholanthrene): 3-methylcholanthrene will also be given to separate groups of mice and studied as above.

SUMMARY

The above are brief outlines of experiments in progress which are designed to throw light on the role of(A) vitamin A deficiency in the production of lung changes in smoke exposed animals and (B) the possible co-carcinogenic effect of the PR8-A influenza virus in smoke exposed and carcinogen treated mice.

Respectfully submitted,

Clayton G. Loosli

Clayton G. Loosli, M.D., Ph.D.
Hastings Professor of
Medicine and Pathology

July 3rd, 1975.

1003540643

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

July 3rd, 1975

Justification of the Budget:

The above is a brief outline of studies in progress and supported by the Council for Tobacco Research - USA. These studies were referred to in progress report VI covering the period of February 1st, 1973 to August 1st, 1974. We estimate that it will take another nine months beyond October 1st, 1975 to complete them and to summarize and publish the data. We believe these studies are highly pertinent to understanding the effects of cigarette smoke inhalation on the lungs of experimental animals and, indirectly, of man.

While the principal investigator is three score and 10, the University Administration has extended the date of retirement in order for him to complete the above. The University's contribution in salaries is estimated to be twice that requested from the Council for Tobacco Research.

The salaries requested are only for the technical personnel. The smoke machine operators are for 6 months while the remaining are for 9 months. The material and supplies are based on the level of past expenditures during the past 3 years.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

BUDGET

OCTOBER 1, 1975 to JUNE 30, 1976

A. Salaries

Professional:	TIME	SALARY	ANNUAL
Clayton G. Loosli, M.D., Ph.D. Principal Investigator	80%	0	0
Edwin B. Howard, D.V.M. Assoc. Professor of Pathology	20%	0	0
John Hardy, M.S. Research Assistant	100%	0	0
Virologist	50%	0	0
Secretary	80%	0	0
Technical:			
Daniel Popenoe, B.S., Histochemistry	50%	50%	*$3,000.00
LaVine Strom, B.S., Histologist	100%	100%	* 6,368.00
Raimonda Apeikis, M.S., E.M. Tech.	50%	50%	* 3,893.00
Carlos Garcia, Animal Caretaker	100%	100%	* 8,476.00
Alicia Navarro, Smoke exposure op.	100%	100%	** 3,946.00
Janna Saghatelian, Smoke exposure op.	100%	100%	** 3,645.00
			$29,328.00

*Nine months
**Six months

Fringe Benefits 12%:	3,519.00
SUB-TOTAL FOR A:	$32,847.00

B. Consumable Supplies (Major categories)

Vivaria	$1,500.00
Food - regular and vitamin A deficient	2,000.00
Fertile eggs	500.00
Histopath., histochem., microbiol.	500.00
Electron microscopy	500.00
SUB-TOTAL FOR B:	$5,000.00

C. Other Expenses (Itemize)

Service and repair of equipment	$1,000.00
Office and communication costs	1,000.00
Publication costs	2,400.00
Travel	600.00
SUB-TOTAL FOR C:	$5,000.00

RUNNING TOTAL OF A, B, C:	$42,847.00
INDIRECT COSTS (15% of ABC):	6,427.00
TOTAL:	$49,274.00

Respectfully submitted,

Clayton G. Loosli, M.D., Ph.D.

7-3-75

10035406 45

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

UNIVERSITY OF SOUTHERN CALIFORNIA
SCHOOL OF MEDICINE
2025 ZONAL AVENUE
LOS ANGELES, CALIFORNIA 90033

IN REPLY, REFER TO
DEPARTMENT OF PATHOLOGY

TELEPHONE
(213) 226-2191

RECEIVED
JUL 11 1975

July 7, 1975

Vincent F. Lisanti, D.M.D.
The Council for Tobacco Research - USA
110 East 59th Street
New York, New York 10022

Dear Dr. Lisanti:

As I said in my letter of July 3rd, I am sending photos of post-influenzal lesions in C57Bl/6J vitamin A deficient mice. This is the first vitamin A experiment in C57Bl mice carried out in the laboratory. They are (Plates I and II) taken from slides sent you for Dr. Sommers' inspection several days ago. These are extremely spectacular, in my opinion, in that the lesions resemble very much those which Nettesheim et al (J. Nat. Cancer Inst. 47: 697-701, 1971) reported in mice following the intra-nasal instillation of 3-methylcholanthrene (MCA). A second experiment is in progress during which the post-influenzal lesions will be observed for a longer period of time and transplants will be made in vitamin A deficient and non-deficient mice.

The post-influenzal hyalinized lesions also resemble those reported by Davis et al (Brit. J. Cancer 31, 1975) in rats following intratracheal instillation of 3-4Benzpyrene (Figs. 2,3,4,5,6 on pp. 448-51), from repeated instillations of tobacco smoke condensates (Fig. 3, p.460), and from inhaled tobacco smoke with or without exposure to 3-4 benzpyrene (Figs. 4&5, p. 474). I am more and more convinced that there is an infectious component to these experimental tumors and that interference with vitamin A function probably plays a role. The studies which we have outlined for the coming year will help answer these questions.

With further reference to the studies of Davis et al, I am not convinced that their interpretation that the alveolar epithelium undergoes cuboidal cell metaplasia is correct. Our studies with Sendai virus infections show that

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

alveolar epithelialization occurs as a result of peripheral growth of bronchial lining membranes which replace the regular alveolar linings. These can be shown by employing G1-6-PDH and LDH enzyme staining procedures which differentiate cells derived from bronchial and alveolar epithelium respectively.

We are beginning to study, by electron microscopy and other means, pulmonary macrophages in the lungs of animals subjected to cigarette smoke inhalation. In a previous report given at the 31st Annual Meeting of the Electron Microscopy Society of America, 1973, crystals in pulmonary macrophages in old mice (CD-1 strain), living in synthetic smog and filtered air, were reported (see enclosed reprint). It was of interest that cytoplasmic crystals were not seen in pulmonary macrophages from animals sacrificed before 12 months of age.

Likewise, cytoplasmic crystals have not been seen in pulmonary macrophages up to 12 months of age in C57Bl/6J, C57L/J and SWR/J mice. Animals sacrificed after this time show varying numbers of crystals and other inclusions in pulmonary macrophages in shelf control, sham control, and smoke exposed mice (C57Bl/6J Plates III to VII), C57L/J (Plates VIII to XIII), and SWR/J (Plates XIV to XVIII). Macrophages containing crystals and other cytoplasmic inclusions were also observed in the peribronchial interstitial tissue of the smoke exposed mice. The cytoplasmic crystals and other inclusions in pulmonary macrophages of smoke exposed mice resemble those recently reported by Brody and Craighead (Lab. Invest. 32(2): 125-132, 1975.)

We have the opportunity to study the nature of the cytoplasmic crystals and amorphous debrae with energy-dispersive X-ray spectrometry similar to that employed by Brody and Craighead. Dr. Taylor, Associate Professor of Pathology and Chief of E.M. and X-ray spectrometry of the Coroner's Office at the LAC-USC Medical Center, will collaborate in carrying out these studies. His laboratory is equipped with apparatus similar to that used by Brody and Craighead. In addition, with the above procedures, we also plan to analyze whole tobacco and cigarette paper, ash from burned cigarettes, and smoke particles collected on cambridge filters.

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What is of interest to us is that intracytoplasmic crystals are a characteristic finding in pulmonary macrophages from old animals whether or not they have been subjected to cigarette smoke inhalation.

As was stated in the progress report VII, pulmonary adenomas were not seen in the three groups of mice sacrificed after cigarette smoke exposure for 12 months. However, some were present in the lungs of the SWR/J mice sacrificed after 19 months of smoke inhalation (June 17th, 1975). They were present in about the same frequency (30 to 40 percent) in the lungs of shelf control, sham control, and smoke exposed vitamin A deficient and non-deficient groups. This indicates to us that the appearance of the pulmonary adenomas is related to the age of the animals and not to cigarette smoke inhalation. A more detailed analysis of the SWR/J lungs is being made. These findings also show the importance of continuing the smoke exposure of the C57Bl/6J and C57L/J mice for another several months.

Sincerely,

Clayton G. Loosli, M.D.
Hastings Professor of
Medicine and Pathology

CGL:ra

Enclosures

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 15, 1975

MEMORANDUM

TO: Drs. Hockett, Jacobson, Meier, Sommers and Stone

FROM: W. U. Gardner

SUBJECT: Grant 573-C.
Progress Report - August 1, 1974 to July 1, 1975.
Request for Extension from October 1, 1975 through June 30, 1976.
Clayton G. Loosli, M.D., Ph.D., Hastings Professor of Medicine and Pathology, University of Southern California School of Medicine, Los Angeles.
"The Effects of Fresh Cigarette Smoke Inhalation on the Respiratory Tract of Mice."

Comment on the Progress Report. The work on the vaccination against the Sendai virus infection is now in press. Four strains of mice have been used -- C57Bl/6J, C57L/J, SWR/J and CD-1. The mice with Sendai virus had lesions like those of mice with PR8-A influenza virus infection. There was, however, no cross reaction between the two viruses.

Mice of all except the CD-1 have been exposed to the smoke of either 1 or 4 cigarettes daily for up to 20 months. Some mice of each group have been on no vitamin A for a number of months. These mice are now all off the experiment. Up to 12-months exposure there had been no bronchial metaplasia or alveolar adenomas. The major change was a chronic pneumonitis. All of the SWR/J mice have been taken off and the usual morphological and surfactin studies are being done.

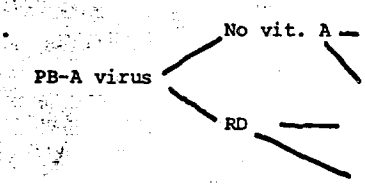
Proposed Continuation.

1. Follow some of the C57- mice mentioned above for four more months or until 26 months of age.

2. Start a group of C57 mice 5- and 10-months old, no vitamin A with regular diet controls then progressing to 6 cigarettes daily for 6 months and then expose to PR-8 in December 1975.

3. Similar to above using younger mice (starting 4 months of age).

4. Study influenza lesions for 40-80 days in no vitamin A C57 mice. The 0-25-day lesions are already available.

5. 

The intratracheal carcinogens will be given to mice under pentobarbital. Each carcinogen and controls will require 250 mice.

Slides have been sent for examination by SCS.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Funds Requested. $49,274.00 For technical assistance for 9 months, animal care for 6 months, expendable supplies and publication costs. Professional and secretarial salaries will be provided from other sources.

Reprint. "Effect of Vitamin A Intake on the Pathology of Airborne Influenza A Virus Infection." Very little difference between no vitamin A, RD and HA except a little more metaplasia in the no vitamin.

Recommendation. Submit to SAB for consideration at September meeting.

W.U.G.

WUG:ek

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

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#891A-NEURATH

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

July 10, 1975

Grant application No. 891A

CANCER

To: The committee comprising Drs. Gardner, Huebner and Sommers

Subject: Dr. Georg B. Neurath, Microanalytical Laboratory, West Germany
Continuation Grant No. 891A
"Nitrosamines in Tobacco and Its Smoke Occurrence, Formation, and Transfer"

History

A three year plan was approved by the SAB in October 1972 to support studies entitled "Kinetics of the Nitrosamine Formation in Tobacco Smoke", with support for the final year (January 1, 1975 through December 31, 1975) of $46,120.

The present proposal, with its change of title, is essentially a continuation of the previous work.

Request

Application No. 891A requests $49,680 for the year 1976, of a two to three year project.

Documents submitted (attached)

1. Application dated July 3, 1975 (7 pages).

2. Progress Report No. 5 for the period January 1, 1975 to June 30, 1975 (11 pages).

3. One reprint (acknowledgement to CTR).

4. One manuscript (19 pages). One of the two papers to be presented to the "Fourth Meeting on Analysis and Formation of N-Nitroso Compounds" organized by the WHO-International Agency for Research on Cancer.

Comment

Dr. Neurath has also submitted an application (Application No. 1035) to support a one year pilot study on "Interaction among Smoking Habit and Drug Bioavailability" requesting $53,130.

D.S.

DS:wg
Atts.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

#2914
Received
July 9, 75

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 8 - 1975

Application For Research Grant
(Use extra pages as needed)

Date: July 3, 1975

1. Principal Investigator (give title and degrees):

Dr. Georg B. Neurath

2. Institution & address:

Microanalytical Laboratory
Hexentwiete 32, 2000 H a m b u r g 56, F.R. Germany

3. Department(s) where research will be done or collaboration provided:

4. Short title of study:

NITROSAMINES IN TOBACCO AND ITS SMOKE
OCCURRENCE, FORMATION, and TRANSFER

5. Proposed renewal date: January 1, 1976

6. How results to date have changed earlier specific research aims:

No changes in the aims. Nitrosamines in t o b a c c o included additionally

7. How results to date have changed earlier working hypothesis:

No changes.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

8. Any additional facilities now required? Describe briefly:

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No changes

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10. Append outline of experimental protocol for ensuing year.
11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. Measurement of nitrogen oxides in tobacco smoke by means of the chemiluminescence method . WHO-IARC Publ. No. 9 (1975) pp. 177-179
2. The nitrosation of nornicotine and nicotine in gas mixtures and aqueous solutions. ibid. in press (see copy of manuscript, enclosed)
3. The interaction of nitrogen oxides, oxygen, and amines in gaseous mixtures Ibid. in press (see copy of manuscript, enclosed)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Chemist	30	$ 8,500
Chemical Engineer	100	$19,000
Technical		
Technician	50	$ 4,600
Sub-Total for A		$32,100
B. Consumable supplies (by major categories)		
Chemicals		$ 3,200
GC- supply and glass		$ 2,900
Small apparates		$ 1,200
Supply gases for GC		$ 2,300
Sub-Total for B		$9,600
C. Other expenses (itemize)		
Travel expenses		$ 1,500
Sub-Total for C		$ 1,500
Running Total of A + B + C		$43,200
D. Permanent equipment (itemize)		
None		
Sub-Total for D		ø
E. Indirect costs (15% of A+B+C)		$6,480
Total request		$49,680

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
ø	ø	ø	

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Dr. Georg B. Neurath

Mailing address for check: Hexentwiete 32
2000 Hamburg 56, F.R. Germany

Principal investigator

Typed Name Dr. Georg B. Neurath

Signature _____ Date July 3, 1975

Telephone Germany(040) 81 6o 4o
Area Code Number Extension

Responsible officer of institution

Typed Name _____

Title _____

Signature _____ Date _____

Telephone _____
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

DR. GEORG B. NEURATH
Vertragsforschung und Beratung

THE COUNCIL FOR TOBACCO
RESEARCH - U.S.A., INC.

110 East 59th Street
New York, N.Y. 10022

2000 Hamburg 56
Hexentwiete 32
Telefon (040) 81 60 40

July 3, 1975

Plan of Work

NITROSAMINES IN TOBACCO AND ITS SMOKE
OCCURRENCE, FORMATION, AND TRANSFER

Hoffmann, Hecht, Ornaf, and Wynder reported in Science (1974) on the occurrence of N-nitroso nornicotine (NNN) in unburned tobacco. The most striking value was reported in a brand of chewing tobacco (C; 88.6 ppm), i.e. 10-30-fold the concentration compared to all other tobacco samples analysed. The artefactual formation of NNN - considered to be conceivable by the authors - was obviously precluded only with two of the samples analysed in this study (cigarette B and chewing tobacco C).

Making use of the experiences collected in preceding studies on the behaviour of all reactants involved in the formation of nitrosamines and on analytical procedures, those results should be controlled.

Although the average content of nitrite in tobaccos is about 40 ppm (Lipp and Dölberg, 1964), the formation of nitrosamines in tobacco under certain conditions seems generally to be possible. The nitrite concentration could be that left after reaction with other tobacco constituents, i.e. also amines, under formation of nitrosamines.

Nitrite in tobacco is probably formed by reduction of nitrate, the concentration of which varies remarkably among tobacco varieties (Neurath and Ehmke, 1964; Lipp and Dölberg, 1964). The nitrite

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

contents of different tobacco products may also vary according to tobacco treatments and ageing processes. Technological steps could influence the formation of nitrosamines, as for instance in the case of chewing tobacco.

It should be studied how and how far preformed nitrosamines would be transferred from tobacco to main and side stream smoke.

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Priority List

1. Determination of volatile and non-volatile nitrosamines in a variety of tobacco products

Making use of the most dependable method - finally accepted method from the running studies - volatile and non-volatile nitrosamines shall be determined in several cigarette, cigar, pipe, chewing tobaccos, and snuff.
Technological influences should be detectable by this survey.

necessary steps:

1.1 Comparison of different extraction methods for the nitrosamines as to artefactual formation. Possible influences can be due to solvent and p_H at extraction.

1.2 Comparison of different methods for gas chromatography detection with mass spectrometry. Chemiluminescence, electron capture, and N-specific detectors shall be compared to mass spectrometry as to specifity.

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

2. Determination of volatile and non-volatile nitrosamines in tobacco varieties

The influence of tobacco growing conditions and fertilizers on the possible formation of nitrosamines in tobacco shall be studied under corresponding aspects to 1.

3. Formation of nitrosamines in tobacco

Facing the results of 1. and 2., it shall be studied, which of the tobacco constituents, and under which conditions and reactions lead to the formation of nitrosamines - nitrite or nitrate content, reduction of nitrate by tobacco constituents as for instance reducing sugars, katalytic effects, or special technological treatment of certain tobacco products, for instance chewing tobacco.

4. Transfer of nitrosamines from tobacco to smoke

Model studies on the transfer rates of volatile and non-volatile nitrosamines from tobacco to main and side stream smoke shall be accomplished by means of ^{14}C-labelled dimethylnitrosamine and N-nitroso nornicotine.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Anticipated duration

The duration of the study described is anticipated to be two to three years.

Estimated position - end of the 1 st year

The estimated position at the end of the first year is that determinations in tobacco products and in tobacco varieties will be completed.

Facilities

Gas chromatography and liquid chromatography of all kinds. Chemiluminescence apparatus. Equipment for tracer studies available.

Administration

Dr. Georg B. Neurath, President

Staff

Four professionals, involved with microanalytical determinations of environmental chemicals, drug metabolism studies and other Trace substances.

Budget, 1 st year	$49,680
Projected, 2 nd year	$53,000

Proposed starting date: January 1, 1976

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

CTR Grant No. 891

Progress Report No. 5
January 1 - June 30, 1975

Dr. Georg B. Neurath
Microanalytical Laboratory

Hexentwiete 32
Hamburg 56, F.R. Germany

KINETICS OF THE NITROSAMINE FORMATION IN TOBACCO SMOKE

Formation of N-nitroso nornicotine from nornicotine and nicotine in model trials

Summary

The formation of N-nitroso nornicotine has been studied from nornicotine and nicotine as free bases in gaseous mixtures comparable to cigarette smoke as to concentrations of the reactants.

Nornicotine as a free base - not protected as salt and retarded by intersolubility and competition with other smoke constituents, as it is in original cigarette smoke - is nitrosated to about 20 % in 5 minutes. The time available for this reaction will be very much shorter in fresh natural smoke.

Of nicotine, as free base and purified from nornicotine, after 15 minutes at room temperature only 0,86 % had reacted to form N-nitroso nornicotine. During a smoke puff followed by in- and exhalation the rate will be smaller by orders of magnitude. At 80°C and 220°C no detectable amounts of N-nitroso nornicotine have been formed. This is obviously due to the negative temperature coefficient of the oxidation constant of nitric oxide and the almost complete cleavage of dinitrogen trioxide (N_2O_3), the nitrosating agent, at those temperatures.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Like many other tertiary amines nicotine is nitrosated in aqueous solutions but in the order to promille during several hours. Obviously two p_H maxima at about p_H 4 and p_H 1 appear.

The contribution of nicotine to the N-nitroso nornicotine content of cigarette smoke appears to be very doubtful. Only very small amounts of N-nitroso nornicotine may be formed due to the nitrosation of nornicotine under realistic conditions in the smoke.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Summary of preceding progress reports

The chemiluminescence determination of nitric oxide in smoke puffs of 40 brands revealed the average content to be of the order of 400 vpm (540 ng/ml) nitric oxide, corresponding to 450 µg/cigt. (8 puffs). Almost no nitrogen dioxide is present in the genuine smoke.

Nitric oxide in model systems as well as in ageing smoke is oxidized to nitrogen dioxide in a pseudo-second order reaction with a half life time of 930 seconds in concentrations and under conditions corresponding to normal smoke.

In model trials nitric oxide is consumed much faster (exactly double the rate) in presence of secondary, and, to a lesser degree, by tertiary, primary amines, and ammonia, but much less it is diminished by alcohols. This enhanced decrease of the nitric oxide concentration is due to the nitrosating reaction: $NO + NO_2 + 2\ R_2NH = 2\ R_2N\text{-}NO + H_2O$, N_2O_3 being the nitrosating intermediate.

In ageing cigarette smoke the declining rate of nitric oxide was found to be of the same order of magnitude as with secondary amines.

Three factors relevant in the smokers respiratory tract strongly influence the half life time of the oxidation of nitric oxide, and probably, as a consequence, the formation of nitrosamines during smoking: 1. Elevated temperature (38°C), for the temperature coefficient for the necessary oxidation of nitric oxide is negative, 2. almost 100 % relative humidity reduce the oxidation rate and, 3. the oxygen content of the smoke-air mixture in the pulmonary alveoli only slightly exceeds the smoke oxygen content because of the competing absorption of oxygen by hemoglobin.

The importance of these factors has revealed from model trials in systems similar to smoke with respect to the concentration by means of the chemiluminescence methods for nitric oxide as well as for nitrosamines.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

In contrary to assumptions discussed recently, nitric oxide is not quantitatively retained in the smokers respiratory tract. The exhalate of the first breath after inhalation with three smokers accounted for between 15 and 45 % of the nitric oxide of a puff.

Very specific measurements of nitrosamines can be achieved by the determination of the nitric oxide formed by thermal decomposition of the nitrosamines and application of the sensitive and selective chemiluminescence method under investigation. If special precautions are observed, this determination can be applied even in presence of oxygen, i.e. to tobacco smoke.

In order to pursue this aim the method has been applied to the effluents of a simple and special gas chromatographic set up, which shall be applied to smoke.

Different trapping systems usually applied to nitrosamine determinations in tobacco smoke have been shown by this means to give rise to remarkably different results.

1003540664

Comparison with data collected from preceding studies on the reaction kinetics and by means of experimentally determined reaction constants of the gas phase reactions involved in the formation of volatile nitrosamines revealed some traps to deliver too small figures, probably due to escaping - alkali and other aqueous traps, - whereas others were shown to cause much higher values than to be expected from kinetic data (solvents/dry ice).

The formation of N-nitroso nornicotine from nornicotine in aqueous solutions had been found to be the highest at p_H 3.8. At p_H 1.5 and lower and at p_H 5.2 and higher almost no reaction occurs.

In 1-molar buffers the reaction rate was found to be smaller than in 0.2-molar buffer solutions.

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Introduction

Papers of Klus and Kuhn (15) as well as Hoffmann, Rathkamp, and Liu (16) on the occurrence of N-nitroso nornicotine in cigarette smoke and a paper of Hoffmann, Hecht, Ornaf, and Wynder (17), who reported the occurrence of N-nitroso nornicotine in unburned tobacco also, have focussed the interest to the formation of that nitrosamine not only from nornicotine but also to that from nicotine. This latter in context with Lijinski's publications dealing generally with the formation of nitrosamines from tertiary bases (18).

This report deals with part of the reaction kinetic studies on the formation of N-nitroso nornicotine (NNN) from nicotine and nornicotine in aqueous solutions and gas phases comparable to cigarette smoke as to concentration of the respective reactants and conditions.

Nitrosation of nornicotine and nicotine in gaseous mixtures by nitric oxide and oxygen.

In order to approach the conditions of nitrosating possible in the tobacco smoke aerosol, similar systems have been applied as already described in Progress Reports 2 and 3.

Two four-necked flasks of 540 ml each, connected with a teflon tube, were evacuated to about 0.1 mm/Hg. The first was filled with nitric oxide test gas of 1000 vpm nitric oxide in nitrogen. Into the second 10 µl nornicotine or 50 µl nicotine (nornicotine content less than 10 ppm) were injected through a septum. The flasks have been opened to each other and through the second flask air has been let in so, that the whole system has been adjusted to atmospheric pressure and to an oxygen concentration of about 10 %, corresponding to cigarette smoke.

Nornicotine and nicotine as free bases participated in the reaction in concentrations according to their vapor pressures at the respective temperatures: 22°C and 80°C. I.e., nitric oxide had been in excess at 22°C, whereas the tobacco alkaloids had been in excess at 80°C.

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The reactions have been pursued by gas chromatographic determination of N-nitroso nornicotine (NNN) after isolation by extraction of the gas mixtures with 10 ml dichloromethane at the listed time intervals. For each measurement a new run had been necessary this way. Completeness of the extraction of NNN has been proved by sequential extraction, which revealed no further NNN to be present in the extracted systems.

The results are summarized in tables 1 and 2 showing, that nornicotine, as free base and not hindered by other components by intersolubility or competition for N_2O_3, i.e. under optimal conditions for the gas phase reaction as to salt/free base-proportion and access of the free base to the gas phase, reacts fairly rapidly like other secondary bases.

Nicotine, however, also forms NNN, but after 15 minutes less than 1 % had been nitrosated at 22°C.
At 80°C and 220°C no formation of NNN from nicotine occurs, which seems to be due to the fact, that according to the negative temperature coefficient of the oxidation of nitric oxide almost no nitrogen dioxide can be formed. Furthermore under these conditions, the amount of NO_2 formed, does not with the abundant NO combine to N_2O_3, the nitrosating agent, because the equilibrium

$$NO + NO_2 = N_2O_3,$$

at those temperatures, is shifted extremely to the left side of cleavage. At 100°C for instance and 0.01 atm. only 0.03 % appear in the form of dinitrogen trioxide; the partial pressure of NO in tobacco smoke, however, is only 0.0005 atm.

In this light the formation of NNN from nicotine in cigarette smoke appears hardly to be a realistic contribution to the nitrosamine content of the smoke facing the short time consumed by puff, in- and exhalation of smokers.

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Table 1

N-Nitroso nornicotine formation from nornicotine in gaseous mixtures of oxidizing nitric oxide at 25°C

time	µg NNN formed	run 1	run 2
5′		375	390
10′		490	502
15′		580	586
30′		686	
60′		765	
16 hours		997	

Table 2

N-Nitroso nornicotine formation from nicotine in gaseous mixtures of oxidizing nitric oxide at 25°C

time	µg NNN formed	% of the nicotine which is nitrosated, calculated from vapor pressure
15′	4.7	0.86
30′	9.5	1.73
60′	15.8	2.88

at 80°C and 220°C ø

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Nitrosation velocity of nicotine in aqueous solutions at different p_H values

For comparison with the results on the nitrosation velocity of nornicotine (see Progress Report No. 4) a similar study was accomplished to the nitrosation reaction of nicotine in aqueous solutions.

Hoffmann, Rathkamp, and Liu (16) reported yields of 1 - 7 % after one hour depending on the p_H value. But the authors did not give data about the purity of the nicotine involved in their study.

In order to avoid interferences with natural nornicotine almost ever present in not specially purified samples of natural nicotine, the raw nicotine was freed from nornicotine by nitrosation and separation of the latter by distillation.

The purity of the resulting nicotine, controlled by gas chromatography was 99.9 %; the final impurity by nornicotine was less than 10 ppm.

Gas chromatographic measurement of the N-nitroso nornicotine (NNN) formed in aqueous solutions at p_H values, actually measured and differing from 1.7 to 6.6, at 22°C, revealed, that after one hour only at p_H 1.7 and p_H 2.5 0.32 and 0.20 promille respectively of the nicotine had been nitrosated. The highest amounts measured in the series were obtained after 8 days at p_H 4.0 - 5.0 with 3.3 promille NNN (see table 3). At longer reaction times, from 21 hours on, there seem to be two p_H maxima corresponding to two different ways of reaction, but this was not pursued so far.

At 80°C the reaction proceeds faster. At p_H 3.8 after 5 minutes already more than 1 % of the nicotine had reacted, the highest values observed being reached after 2 hours with 8.49 % NNN formed from nicotine (see table 4).

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Table 3

N-Nitroso nornicotine (promille of nicotine reacted) at 22°C at different p_H values

time	pH					
	1.7	2.5	4.0	4.2	5.0	6.6
30′	0.21	0.10	ø	ø	ø	ø
60′	0.32	0.20	ø	ø	ø	ø
2 h	0.81	0.28	trace	trace	trace	ø
4 h	0.87	0.46	trace	trace	trace	ø
21 h	1.13	0.49	1.4	1.4	1.0	trace
28 h	--	--	2.1	1.7	1.1	-
2 days	1.21	0.49	2.2	1.6	1.8	trace
3 days	--	--	2.3	2.3	2.3	-
4 days	1.38	0.48	-	-	-	0.21
8 days	1.41	0.51	3.3	3.3	3.3	0.32
13 days	--	--	3.0	3.0	3.0	--

Table 4

N-Nitroso nornicotine (% of nicotine reacted) at 80°C at p_H 3.82

time	p_H 3.82
5′	1.23
15′	3.15
30′	4.88
45′	6.06
60′	6.42
100′	7.81
120′	8.49
150′	8.49
180′	8.49
210′	8.49
300′	8.49
48 h	6.0

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Nitrosation conditions in aqueous solutions

About 9 ml of the respective buffer solutions (0.2 molar) 100 µl specially purified nicotine, and 0.5 ml 1n-sodium nitrite solution were stirred at 22°C or 80°C.
The actual p_H values of the reaction solutions were measured by means of an electrometric p_H-meter equipped with a glass electrode.

At time intervals, given in tables 3 and 4, 0.5 ml samples of the solution were taken, basified with aqueous potassium carbonate solution and extracted with 100 µl dichloromethane.
2 µl of the dichloromethane solution were injected for the gas chromatographic determination.

This extraction procedure was compared to the extraction at the respective p_H values of the reaction solution. No difference was found due to the p_H value of the extracted solution.

Gas chromatographic procedure:

Device: Hewlett-Packard hp 5751 G. 1.50 m glass beads, washed with 1 % Silar 5CP in chloroform.
Column: temperature program 70-120°C, 4°C/min, injection port 270°C, FID 330°C, 30 ml nitrogen/min, 30 ml hydrogen/min, 500 ml air/min.

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Nicotine purification

15 g nicotine dissolved in 1 n hydrochloric acid were brought to p_H 3.65 by adding a buffer solution. About 2 g sodium nitrite were added and the solution stirred for 36 hours. After acidifying with 10 % hydrochloric acid, the mixture was extracted three times with dichloromethane. The remainder was basified with 50 % potassium hydroxide solution and extracted with ether. After removing of the ether the nicotine was purified by distillation in vacuum: Kp_{15} 119°C.

No detecable traces of nornicotine appeared in the gas fractogram.

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Literature

(15) H. Klus and H. Kuhn
Fachl. Mitt. österr. Tabakregie 14 (1973) 251-257

(16) D. Hoffmann, G. Rathkamp, and Y.Y. Liu
in P. Bogovski and E.A. Walker
N-Nitroso Compounds in the Environment, IARC Scientific Publication No. 9, Lyon (1975) 159-165

(17) D. Hoffmann, S.S. Hecht, R.M. Ornaf, and E.L. Wynder
Science 186 (1974) 265-267

(18) W. Lijinski and G.M. Singer
in P. Bogovski and E.A. Walker
N-Nitroso Compounds in the Environment, IARC Scientific Publication No. 9, Lyon (1975) 11-114

Literature is numbered through all progress reports on "Kinetics of the Nitrosamine Formation in Tobacco Smoke"

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#1005A-WANG

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 24, 1975

Grant Application No. 1005A

CANCER

TO: The committee comprising Drs. Huebner, Jacobson and Lynch

SUBJECT: Irene Y. Wang, Ph.D., Medical University of South Carolina, Charleston, S.C.
Continuation application No. 1005A
"Genetic Differences in the In Vitro Metabolism of Chemical Carcinogens by Human and Mouse Tissues"

History

In October 1974 the SAB considered the Wang request for a two year project ($68,284 for the first, and $73,600 for the second year). This study was approved at a level of $65,926 for the period January 1, 1975 through December 31, 1975, without assurance of further support, and contingent upon priority of effort to be given to the mouse studies.

At that time Dr. Wang was located at the University of California, San Francisco. In May 1975, CTR was notified that Dr. Wang had accepted a position in Charleston, S.C., and a request was made to CTR for permission to transfer the grant to the Medical University of South Carolina: CTR approved the transfer -- effective July 1, 1975.

Request

Continuation application No. 1005A requests $47,163. (See comments, below, for additional request)

Documents Submitted (attached)

1. Application dated July 11, 1975 (6 pages).

2. Progress Report No. 1 for the period January 1, 1975 to June 30, 1975 (20 pages including diagrams and tables).

3. One paper: 3 abstracts.

Comments

1. Dr. Wang requests use of $16,000 from Grant No. 1005 (first year support) for purchase of a scintillation counter, fraction collector, nitrogen evaporator and a shaker-incubator -- items urgently needed for research activities to commence immediately. A significant portion of this sum (estimated by Wang at $11,000) will be replaced by State support for both her salary (6 months in 1975) and some supplies.

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2. This copy of the application is not signed by an official of the Institution: the signed original is expected to be forwarded to CTR within a short time.

David Stone

DS/lp
Encls.

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1005A

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8585

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Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☐ Second Renewal ☐ Date: July 11,1975

1. Principal Investigator (give title and degrees):

Irene Y. Wang, Ph.D.
Assistant Professor

2. Institution & address:

College of Medicine
Medical University of South Carolina
80 Barre Street, Charleston, S. C. 29401

3. Department(s) where research will be done or collaboration provided:

Department of Basic and Clinical Immunology and Microbiology

4. Short title of study:

Genetic Differences in the In Vitro Metabolism of Chemical Carcinogens by Human and Mouse Tissues.

5. Proposed renewal date: Jan. 1, 1975

6. How results to date have changed earlier specific research aims:

Yields of individual metabolite of benzo(a)pyrene (BaP) catalyzed by microsoma enzymes of C57B1/6 (B6) and C3H mice are increased to different extents upon pretreatment with BaP, with 7,8-diol being generally increased the most in tissues tested which include liver, lung, spleen and kidney. For DBA/2 (D2) mice, pretreatment with BaP has no effect on liver, the induced enzyme activity in extrahepatic tissues of D2 mice are still lower than those of the corresponding tissues of untreated B6 or D2 mice. The preferential increase in the yield of 7,8-diol may be significant in the tumoregenesis in lungs of susceptible mice, since we reported (1) that 7,8-diol can be further activated by hamster liver microsomal enzymes to bind DNA. It is proposed to add the study of microsomal enzyme activity in various mouse tissues, especially lung, in further activating 7,8-diol to reactive forms.

7. How results to date have changed earlier working hypothesis:

The results to date tend to strengthen the original working hypothesis that there may be a correlation between the metabolite patterns of polycyclic aromatic hydrocarbons(PAH) and the susceptibility of animals to PAH-induced tumoregenesis. In the case of BaP metabolism by different strains of mice, the greater increase in the yield of 7,8-diol may be very important, and this aspect needs to be further studied as proposed above.

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8. Any additional facilities now required? Describe briefly:

Fraction collector: for collecting DNA fractions after CsCl gradient centrifugation of DNA incubated with metabolites of ^{3}H-BaP or of ^{3}H-3-MC.

Nitrogen evaporator: originally two nitrogen evaporators were available in handling large number of sample each time. Dr. Rasmussen transferred one of them to U.C. Irvine.

Cigarrette-smoking machine: for testing effect of cigarrette smoke inhalation on mouse tissue microsomal enzyme activities in produing various metabolites of BaP or 3-MC.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Due to transfers of professionals involved in the original proposal, Dr. R. E. Rasmussen and Dr. N. L. Petrakis are not participating the the continuation of this project.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. Wang, I. Y., Rasmussen, R. E. and Crocker, C. C., Metabolism of benzo(a)pyrene by microsomes from tissues of pregnant and fetal hamsters. Life Science 15, 1219 (1974).

2. Wang, I. Y., Metabolism of benzo(a)pyrene by tissue microsomes from different strains of mice. Proc. Am. Assoc. Cancer Res. 16, 377 (1975).

3. Rasmussen, R. E. and Wang, I. Y., Metabolism and binding of ^{3}H-benzo(a)pyrene to DNA in the presence of liver enzymes from rats treated with benzo(a)pyrene or phenobarbital.

4. Wang, I. Y., Rasmussen, R. E., Petrakis, N. L., Jan, C. C. and Wang, A. C. Enzyme induction and the difference in the metabolite pattern of benzo(a)pyrene produced by various strains of mice. Symposium on Polynuclear Aromatic Hydrocarbons. Battelle Columbus Lab. Oct. 1975.

12. Summary progress report (append in standard form as separate document, unless recently submitted).

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Irene Y. Wang, Ph.D.	40%	----
An-Chuan Wang, Ph.D.	5%	----
Technical		
Lab specialist I (to be recruited)	100%	11,760.00
Lab Specialist I (to be recruited)	100%	11,760.00

(All salaries include 8% increase for projected cost of living and merit raises).

Sub-Total for A 23,520.00

B. Consumable supplies (by major categories)	
Animals	3,000.00
Radioisotopes	2,500.00
Glassware	500.00
Chemicals (NADPH, etc.)	3,000.00
Scintillation counting vials	3,000.00
TLC plates	300.00

Sub-Total for B 12,300.00

C. Other expenses (itemize)	
Medical illustration and publication	1,000.00
Equipment maintance, contract and contracting	1,000.00
Travel	1,500.00
Misc. office supplies	300.00

Sub-Total for C 3,800.00

Running Total of A + B + C 39,620.00

D. Permonent equipment (itemize)	
Nitrogen Evaporator	500.00
Gilson microfraction collector	1,100.00

Sub-Total for D 1,600.00

E. Indirect costs (15% of A+B+C) 5,943.00

Total request 47,163.00

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Role of Metaboli Activation in Chemical Carcinogenesis	South Carolina State Approperiation for Research	$5000.00	July 1, 1975 to June 30,. 1976.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Irene Y. Wang, Ph.D.

Signature Irene Wang Date July 11, 19

Telephone 803 792-4421
Area Code Number Extension

Checks payable to

Medical University of South Carolina

Mailing address for checks: Mr. L. W. Michaelis
Director of Business Operations
Medical University of South Carolina
80 Barre Street
Charleston, South Carolina 29401

Responsible officer of institution

Typed Name Mr. John E. Wise

Title Vice President and Treasurer

Signature _____ Date _____

Telephone 803 792-4291
Area Code Number Extension

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10. Experimental Protocol for the Ensuing Year:

(1) Genetic studies on the metabolite patterns of BaP catalyzed by lung homogneates from C3H, D2 and B6; their cross and backcrosses.

Studies on the genetic differences in the metabolite pattern of BaP by liver homogenates of the above strains of mice are in progress. The optimal incubation conditions for the production of diols of BaP is being set up, since the precusors of the diols -- epoxides of BaP, or 7,8-diol itself after further metabolic activation may be the ultimate carcinogens of BaP. Data to date indicate that for liver homogenate incubation system of C3H, D2 or C3HxD2 mice, short incubation interval (e.g. 3 min.) and low homogenate protein concentrations (0.25-0.5 mg/ml) are favorable for avoiding secondary metabolism of BaP metabolites. Different tissues have different kinetic curves for the metabolite yields of BaP. Best incubation conditions using lung homogenates of mice will be determined, then host control of metabolite patterns of BaP will be studied.

(2) Further metabolic activation of BaP metabolites by mouse tissue preparations --- DNA-binding in vitro.

We reported previously that 7,8-diol can be further activated by hamster liver microsomes to bind DNA in vitro (1). Recently Sims et al (2) also reported that metabolic activation of BaP proceeds by a diol-epoxide. Therefore, we propose to study the enzyme activities in mouse lung and liver tissue preparations (homogenates or microsomes) in further activating metabolites of BaP (especially 7,8-diol) to active intermediates as indicated by extent of binding to DNA.

Radioactive metabolites of BaP will be prepared from large-scale incubation of ^{3}H-BaP or ^{14}C-BaP using liver microsomes from rats pretreated with 3-MC for 9,10-diol and 7,8-diol, and from control hamsters for 4,5-diol (3, 4). Other metabolites of BaP can be obtained with either rat or hamster liver microsomes as enzyme source. Metabolites of BaP will be separated by TLC as described before (4). For DNA-binding studies, 200 μg prepurified DNA in citrate buffer solution will be incubated with isolated radioactive metabolite of BaP in the presence of NADPH and mouse tissue preparations, at 37°C for 30 to 60 min. Incubation mixtures will then be extracted extensively with mixture of chloroform and isoamyl alcohol, then the aqueous phase dialyzed against citrate buffer solution, and subjected to CsCl density gradient centrifugation. Fractions will be collected and aliquoat from each fraction will be counted for radioactivity as indication of extent of DNA-binding of metabolite of BaP after further enzymic activation.

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(3) Effect of inhalation of cigarette smoke by mice on the metabolite patterns of BaP in vitro:

Cigarette-smoke inhalation by mice will be performed in similar ways as reported by Kouri et al (5), whose results indicated that pulmonary AHH activity of B6, C3H and D2 strains of mice could be induced about six hours post inhalation of one to seven cigarette consecutively. Metabolite patterns of BaP by mouse liver or lung tissue preparations after cigarette-smoke inhalation will be compared for possible correlation the production of certain metabolite(s) of BaP and the susceptibility of lung tissues to BaP-induced tumors.

(4) Modification of metabolite patterns of BaP by fractions of cigarette-smoke condensate:

Some fractions of cigarette-smoke condensate have been reported to induce pulmonary AHH activity, and also inhibit BaP metabolism in vitro, e.g., B_1a, B_1b and N_{NM} (5). Metabolite patterns of BaP and DNA-binding by BaP in incubation system employing mouse tissue preparations will be studied in paralel with the presence of different fraction of condensate in the incubation mixture.

(5) Identification of metabolites of 3-MC catalyzed by liver and lung microsomes form C3H, B6 and D2 mice.

Incubation system for metabolism of 3-MC will be similar to that used for BaP, and extraction as well as separation of metabolites on TLC plates will be carried out as for BaP. UV spectrum of each isolated metabolite of 3-MC will be used for identification.

References:

(1) Borgen, A., Darvey, H., Castagnoli, N., Crocker, T., Rasmussen, R. and Wang, I., J. Med. Chem., 16, 502(1973).
(2) Sims, P., Grover, P. L., Swaisland, A., Pal, K. and Hewer, A., Nature, 252, 326(1974).
(3) Rasmussen, R. E. and Wang, I. Y., Cnacer Res., 34, 2290(1974).
(4) Wang, I. Y., Rasmussen, R. E. and Crocker, T. T., Biochem. Biophys. Res. Commun. 49, 1142(1972).
(5) Kouri, R. E., Demoise, C. E. and Whitmire, C. E., Exp. Respiratory Carcinogenesis and Bioassays, Springer-Verlag, 1974.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Progress Report: Jan. 1, 1975 to June 30, 1975.
CTR Grant #1005
Irene Y. Wang, Ph.D.
Dept. Basic and Clinical Immunology and Microbiology
Medical University of South Carolina
Charleston, South Carolina 29401

Genetic Differences in the In Vitro Metabolism of Chemical Carcinogens by Human and Mouse Tissues.

A. Results:

(I) Effect of BaP-pretreatment on the in vitro metabolite pattern of BaP by mouse tissue microsomes.

To date, five strains of mice have been tested: C57Bl/6 (B6), C3H, DBA/2 (D2), B6xD2 and C3HxD2. Mice were pretreated with BaP (2 mg/100 g body weight) 24 hrs before sacrifice, control mice were injected with corn oil only. Microsomes were prepared from liver, lung, kidney and spleen, protein concentrations were determined by Lowry's method(2). Metabolism of BaP was studied with freshly prepared microsomes. Incubation system consisted of 1 μg/ml ^{3}H-BaP (specific activity 25 ci/mmole), 1 mg/ml NADPH, 0.25 mg/ml microsomal protein in sodium citrate buffer (1). Incubation was carried out at 37°C from three to ten minutes under subdued light. Incubation mixture was extracted twice with 2 volume ethyl acetate, and the organic layer was dried under nitrogen. The residue was redissolved in acetone-ethyl acetate mixture and spotted on TLC plate. Separation of metabolites of BaP was done by chromatography with benzene alone for the detection of BaP-4,5-epoxide, or first with benzene then immediately in the same direction with benzene:ethanol(95:5). Identification of metabolites of BaP was by comparison with authentic compounds co-chromatographed with samples, radioscannings as well as by UV absorption spectra of metabolites separated from large-scale incubation mixtures. Quantitation of metabolites of BaP was made by scintillation counting of strips containing radioactive spots.

(a) Qualitative comparison of metabolite patterns of BaP.

Figs.1, 2, and 3a show the separation of metabolites of BaP catalyzed by liver microsomes from D2, C3H and B6 mice respectively. The metabolites are: peak (a), origin of TLC and more-polar metabolites of BaP; peak (b), 9,10-diol; peak (c), 7,8-diol; peak (d), 4,5-diol; peak (e), 3-OH and other phenols of BaP; peak (f), not identified; peak (g), quinones of BaP; peak (h), BaP-4,5-epoxide; peak (i), unmetabolized BaP.

With liver microsomes of D2 mice, relative peak heights for control and pretreated mice (Fig. 1) are almost identical. With C3H (Fig. 2) and B6 (Fig. 3a) mouse liver microsomes, every

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metabolite was increased due to pretreatment with BaP. The largest increase was at the peak for 7,8-diol. Fig. 3b. shows the separation of BaP-4,5-epoxide from BaP and other metabolites of BaP on TLC plate by chromatography with benzene only.

(b) Quantitative comparison of metabolite yields of BaP.

In Figs. 4, 5, 6, 7, the hedged columns represent results from control mice, and the hollow bars the results from BaP-pretreated mice). As can be seen in Fig. 4, which includes the results from B6xD2 and C3HxD2 mouse liver microsomal incubation systems also, livers of B6 and B6xD2 mice showed great response to hydrocarbon-induced enzyme activities in metabolizing BaP, while increase in the yields of BaP metabolites in pretreated C3H mouse liver was smaller, and D2 mouse liver did not respond to pretreatment with BaP in the enzyme activities in metabolizing BaP. Results to date with C3HxD2 mouse liver microsomes are not clear cut, more studies on individual mouse of this hybrid atrain will be carried out. However, it is very obvious that the greatest increase due to pretreatment with BaP was the yield of 7,8-diol, in the cases with B6, B6xD2 and C3H mouse livers.

For extrahepatic tissue microsomal incubation system for metabolizing BaP, protein concentration was 1.0 mg/ml and incubation for 10 min. All the other conditions were the same as for liver microsomal incubation system. As can be seen in Table 1, 2, or 3, (or Fig. 5, 6 or 7, for comparison of yields of metabolites of BaP by extrahepatic microsomes), metabolite patterns of BaP by C3H and B6 lung microsomes were very similar, as well as the effect on BaP-pretreatment on the enzyme activities in metabolizing BaP by these lung microsomes. D2 strain of mice showed very little response to BaP-pretreatment, there was a small increase in metabolite yields of BaP by lung microsomes of pretreated D2 mice, but the yields were still much lower than those produced by lung microsomes of control B6 or C3H mice. The significance of the preferential increase in the yield of 7,8-diol by lung microosmes of B6 or C3H mice warrent further detailed studies.

(2) Incubation system with homogenates of mouse livers:

Frozen mouse liver pieces were supplied by Dr. R. E. Kouri. Each liver piece was weighed and homogenized in 20 times of TKM-sucrose buffer (1). Protein concentrations were determined by Lowry's method(2). Each incubation sample contained one to four mg/ml homogenate protein in 1 ml TKM-sucrose buffer, 1 μg/ml ^{3}H-BaP (specific activity 25 ci/mmole) in 50 μl acetone, 1 mg/ml NADPH, sodium citrate buffer (1) was added to make the final incubation volume to 2 ml. Incubation was at 37°C for 3 min. Extraction, separation, identification and quantitation of metabolites of ^{3}H-BaP were carried out in the same manner as for microsomal incubation system.

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Fig. 8 shows that metabolite patterns of BaP catalyzed by liver homogenates from control C3H, D2 and C3HxD2 mice are similar. At higher protein concentration (4 mg/ml), yields of quinones of BaP increased at the expense of phenols of BaP. Therefore, lower protein concentrations are preferred in avoiding secondary metabolism of metabolites of BaP. For Fig. 9, 1 mg/ml homogenate protein was used in incubation system. For mice pretreated with 150 μg 3-MC, livers of D2 mice did not show response to pretreatment; while C3H and C3HxD2 mouse livers showed similar response to 3-MC-pretreatment. Yield of 7,8-diol was increased to about 25-fold while that of 3-OH-BaP to about 4-fold.

Studies are in progress in determining the linear portion of the protein concentrations in the production of the diols for genetic studies using crosses and backcrosses of these strains of mice.

(3) Separation of Metabolites of 3-methylcholanthrene (3-MC):

Test runs were done by using microsomes from 60-day old Sprague Dawley rats, since we reported that BaP-pretreatment and phenobarbital (PB)-pretreatment caused different increase in the various metabolites of BaP (3). Incubations were similar to those used for BaP, 10 nmole/ml 3-MC was used in the incubation mixture and incubation was carried out for 10 min. Fig. 10 shows the radioscans of metabolites of 3-MC separated on TLC plate using the same solvent systems as for the separation of BaP metabolites. The metabolites of 3-MC need to be identified, however, this TLC system seems feasible for the separation of 3-MC metabolites catalyzed by mouse tissue microsomes or homogenates. Liver microsomes from PB-pretreated rats seemed to give higher yield of metabolite at position marked x in Fig. 10. Metabolite patterns of 3-MC by mouse liver microsomes will be studied in similar manner in the remaining portion of the current year.

(4) Metabolism of ^{3}H-BaP by Human Lymphocytes in Culture:

This protion of study was proposed in original grant proposal and was instructed to give priority to studies on mouse tissues upon approval of funding of this grant. The data reported here were results obtained previously. Lymphocytes prepared from human peripheral blood were cultured at 2×10^{6} cells/ml in RPMI 1640 medium supplemented with fetal calf serum and anti biotics. For mitogen stimulated lymphocytes, phytohemaagglutinin (PHA) or pokeweed mitogen(PWM) was added in strength of 0.1 ml/10 ml medium at the start of culture. ^{3}H-BaP 10 μci/ml, 25 ci/mmole) was added 24 hrs later. Culture was continued for another 24 or 48 hrs. Medium was then extracted with 2 volumes ethyl acetate and metabolites of ^{3}H-BaP were analyzed in the same manner as described previously. Metabolite patterns of ^{3}H-BaP by lymphocytes in culture 48 hrs after addition of ^{3}H-BaP are shown in Fig. 11, where peak (1),

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origin of TLC and more-polar metabolites of BaP; peak (2), not identified; peak(3), 9,10-diol; peak(4), 7,8-diol; peak(5), 4,5-diol; peak(6), 3-OH and other phenols of BaP; peak (7), quinones of BaP; peak(8), unmetabolized BaP. Panel A of Fig. 11 shows metabolite pattern of ^{3}H-BaP without any mitogens added to the culture medium; panel B shows that the presence of PHA in culture medium caused increase in the yield of phenols of BaP and decreased yields of diols. The quantitative data for the metabolite yields of BaP by lymphocytes in culture are shown in Fig. 12. No further studies on lymphocytes have been undertaken.

B. Plans for the remaining portion of the current year:

(1) Finish analysis data for optimal conditions for metabolism of BaP by liver homogenates, and then study the metabolite patterns of BaP with crosses and backcrosses of C3H, D2 and B6 strains of mice for genetic control of response to enzyme induction for the production of certain metabolites of BaP, especially the yields of the diols.

(2) Study the effect of pretreatment with various inducers of AHH activity on the metabolite patterns of BaP, e. g., the pretreatment of mice with phenobarbital, benzoflavones, fractions of cigarette smoke condensate.

(3) Column chromatography for different forms of cytochrome p-450, employing the method of Comai and Gaylor(4).

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4. Comai, K. and Gaylor, J., J. Biol. Chem., 248, 4947(1973).

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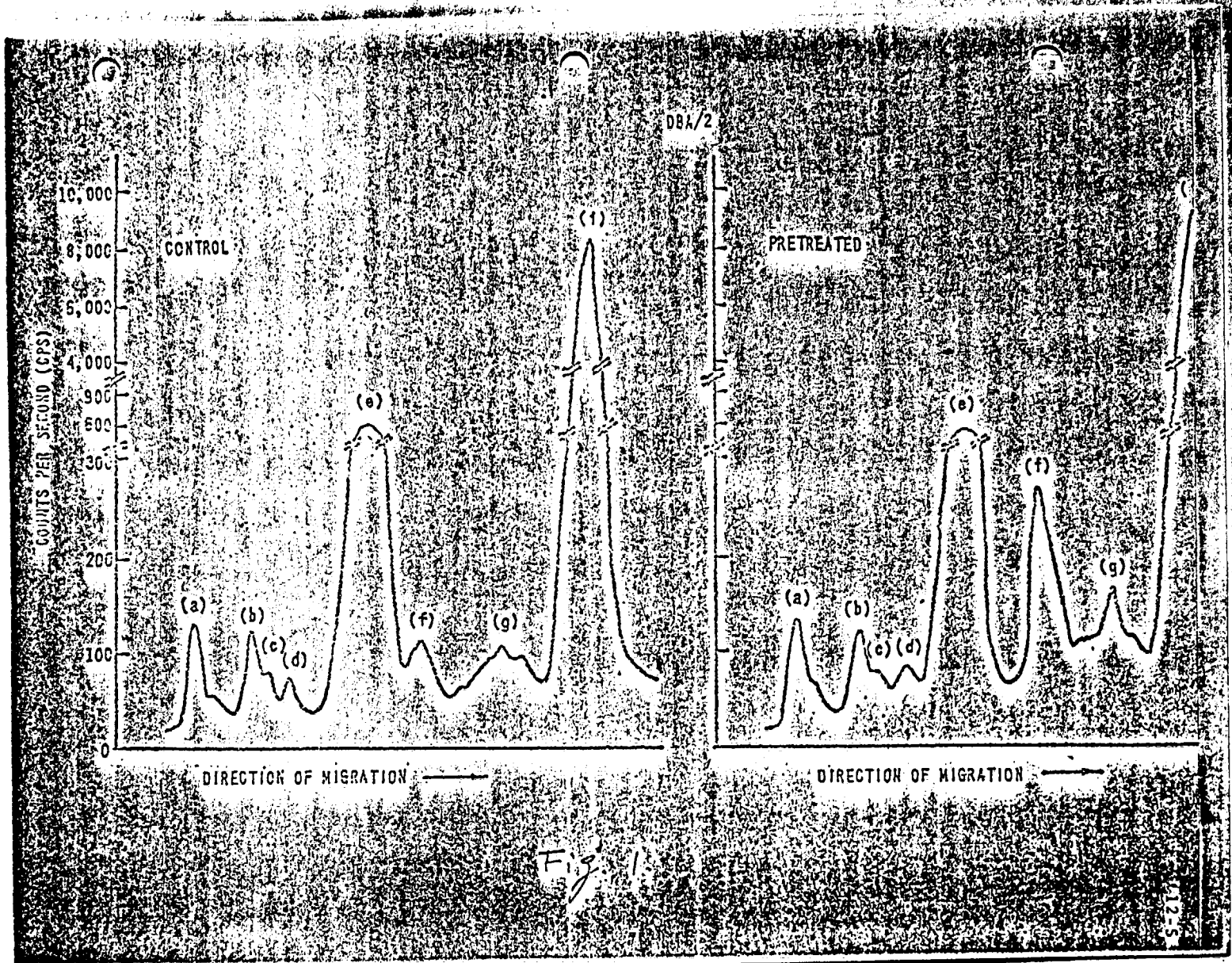

Fig. 1

12-5

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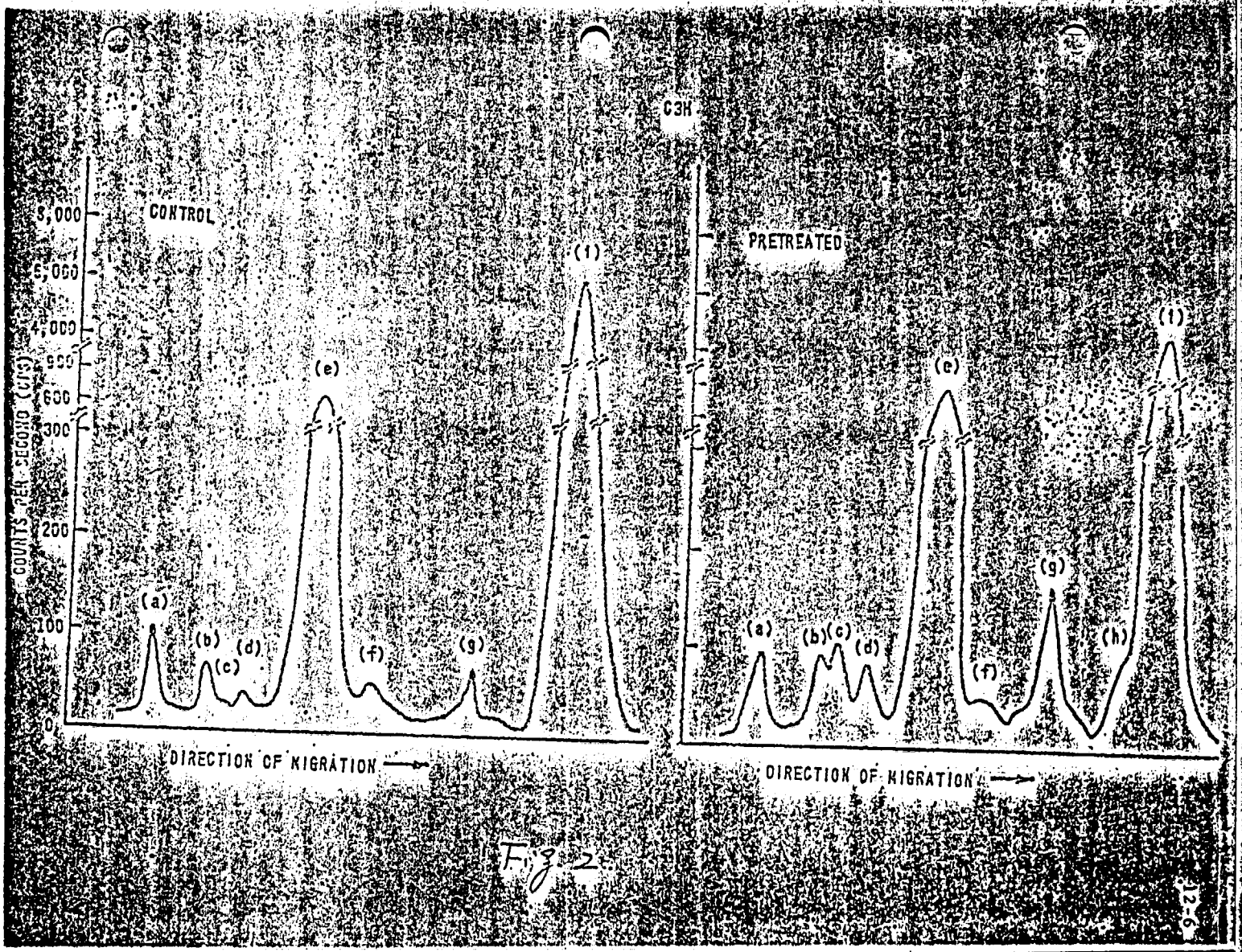

Fig 2

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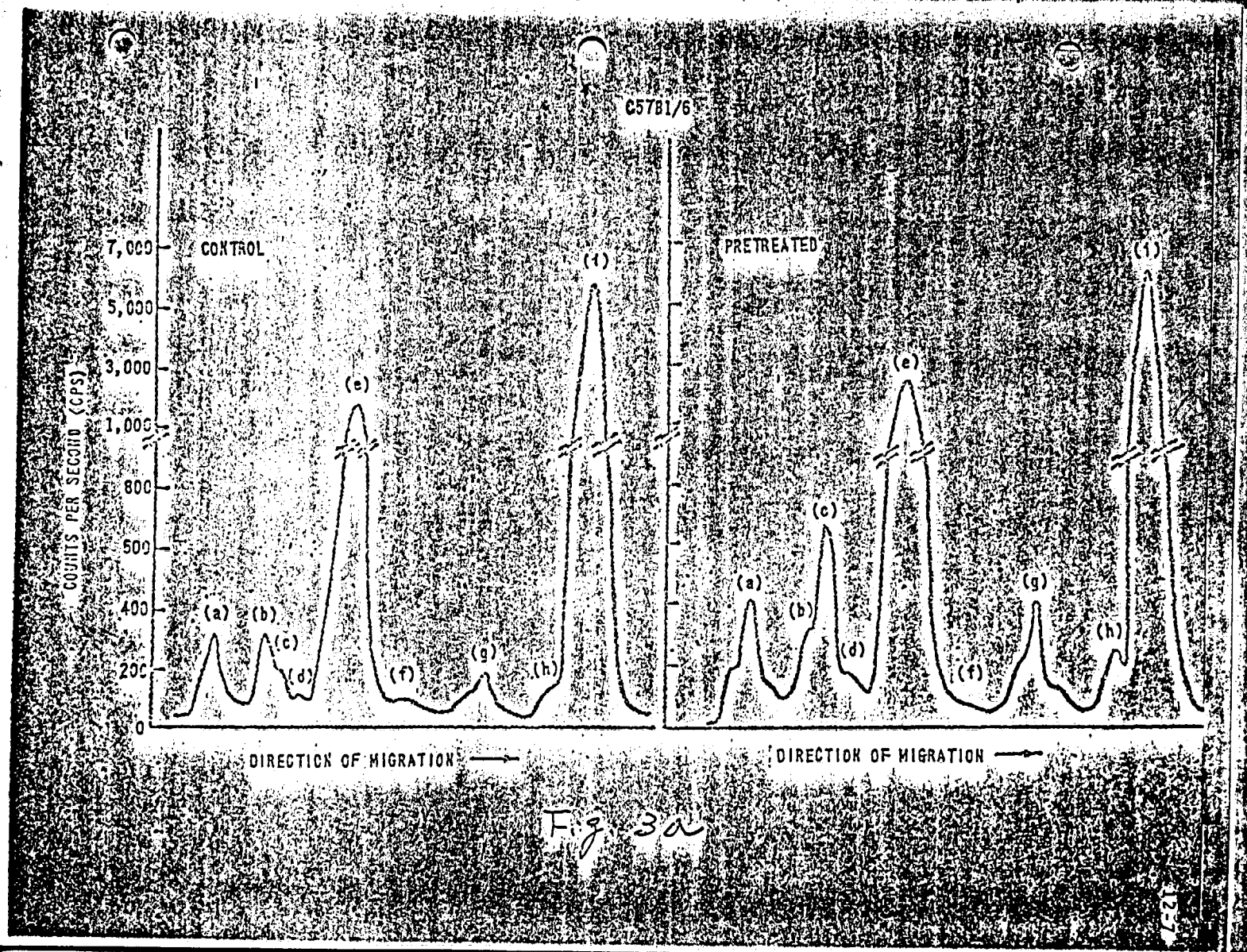

Fig. 3a

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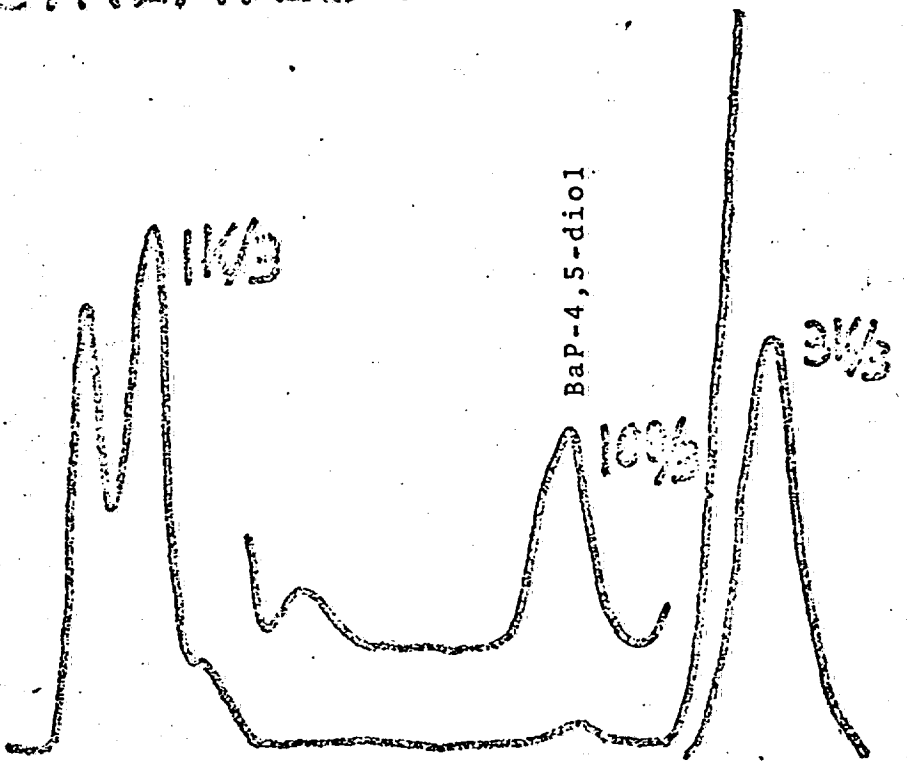
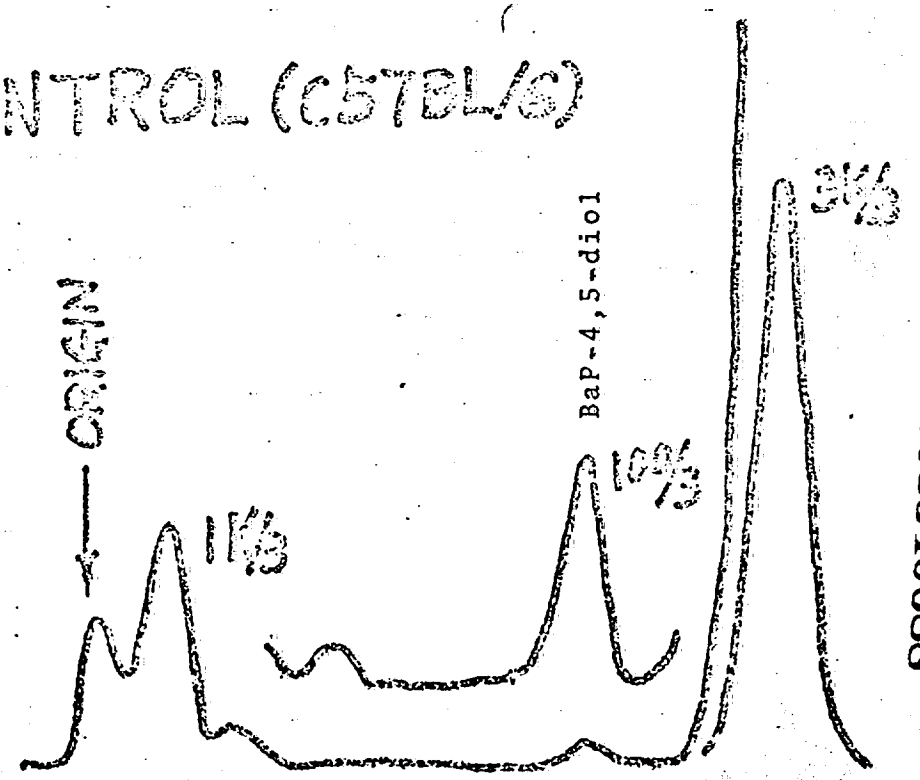
PRETREATED (C57BL/6)



CONTROL (C57BL/6)



DIRECTION OF MIGRATION →

Fig 3b.

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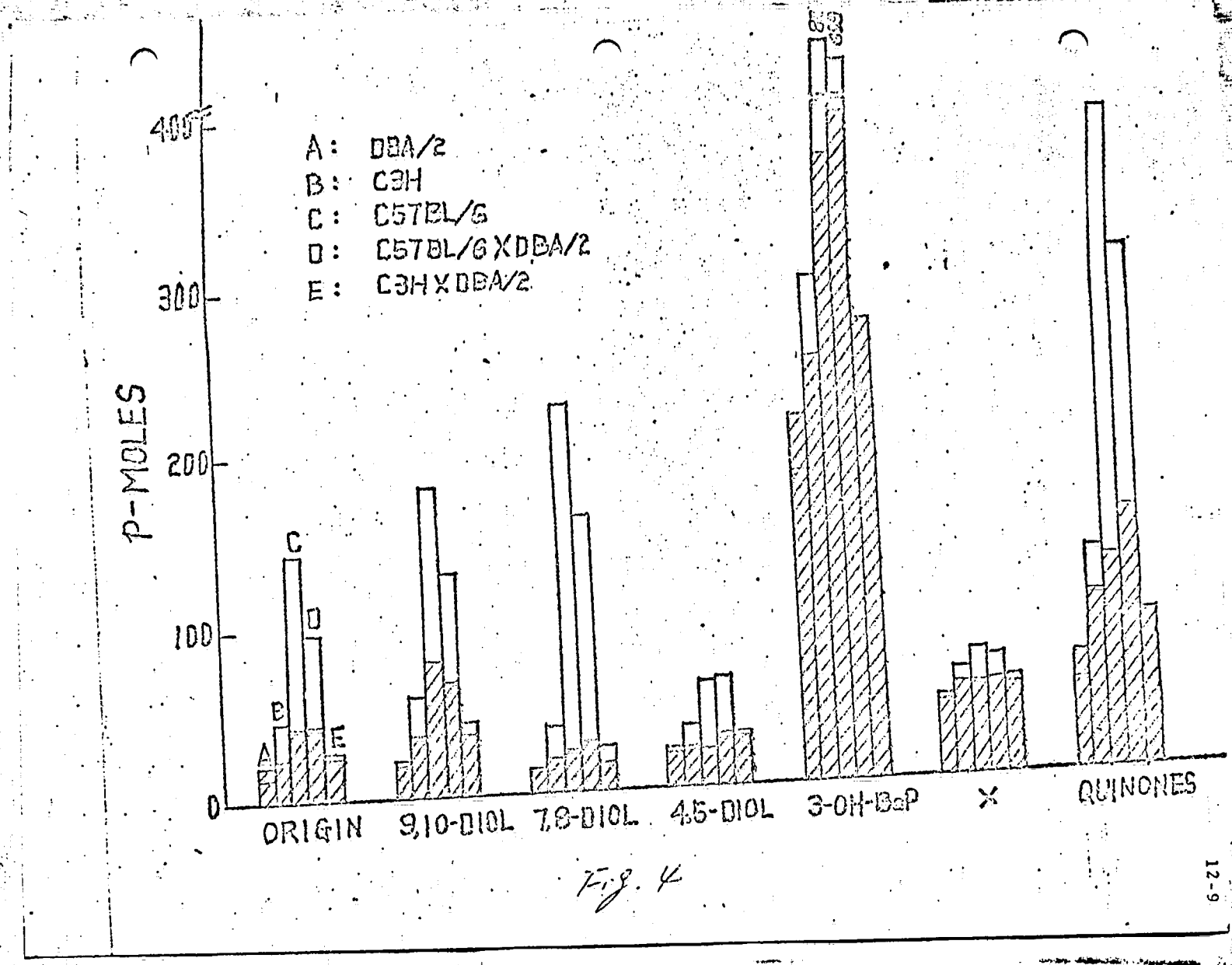

Fig. 4

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TABLE 1.

DBA/2 MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	1.08	3.34	.59	.89	.95	1.58	2.03
9,10-diol	2.14	3.44	2.05	1.19	1.29	2.01	2.69
7,8-diol	2.97	8.82	2.20	1.34	2.18	4.43	1.12
4,5-diol	1.51	7.70	1.02	.84	1.02	.79	1.53
3-OH and other phenols of BaP	9.43	23.74	4.50	6.63	12.33	12.19	4.80
Quinones of BaP	90.28	91.80	99.37	96.74	102.27	98.91	73.98
Totals	107.42	138.84	109.74	107.64	120.06	119.90	86.16

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TABLE 2

C3H MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	2.66	46.40	.61	1.66	.61	1.21	.69
9,10-diol	16.02	126.47	.37	10.77	.70	7.35	.24
7,8-diol	21.40	283.94	.73	12.73	2.92	12.72	.28
4,5-diol	4.39	62.35	.24	2.74	.76	1.35	.37
3-OH and other phenols of BaP	87.01	987.10	5.28	64.88	11.72	35.50	1.51
Quinones of BaP	38.00	349.29	8.33	22.33	15.99	19.66	9.10
Totals	169.48	1855.55	15.56	115.11	32.70	77.79	12.19

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TABLE 3

C57B1/6 MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	5.07	42.04	7.44	11.98	2.07	3.75	1.51
9,10-diol	32.53	248.85	4.45	22.67	3.15	7.56	.47
7,8-diol	25.70	220.11	3.13	53.67	3.67	11.05	.58
4,5-diol	5.51	28.65	3.89	21.59	1.19	2.43	.93
3-OH and other phenols of BaP	121.62	1146.49	17.97	153.66	26.45	63.19	3.27
Quinones of BaP	52.87	338.52	153.12	191.86	40.12	42.38	48.75
Totals	243.30	2024.66	190.00	455.43	76.65	130.36	55.53

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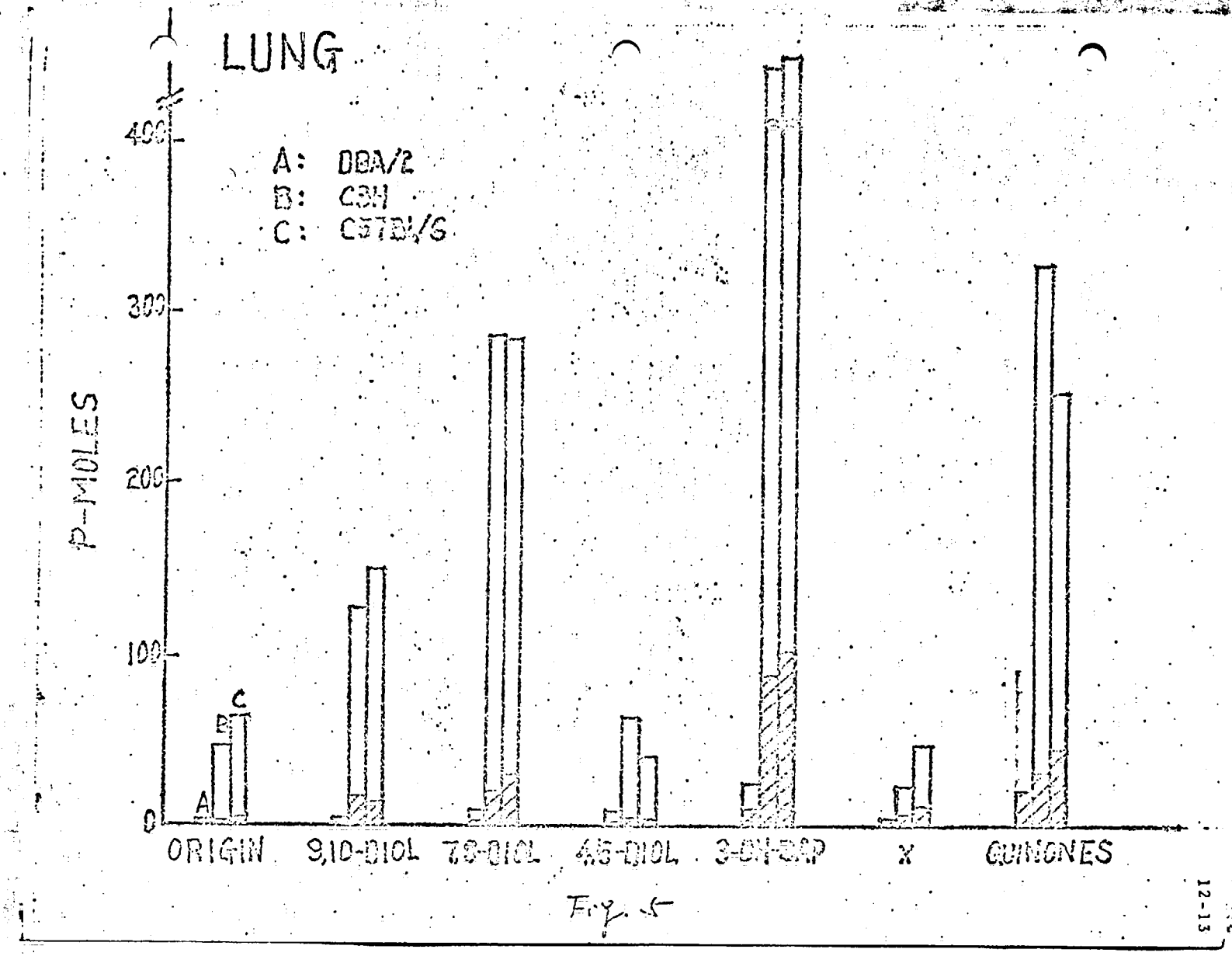

Fig. 4

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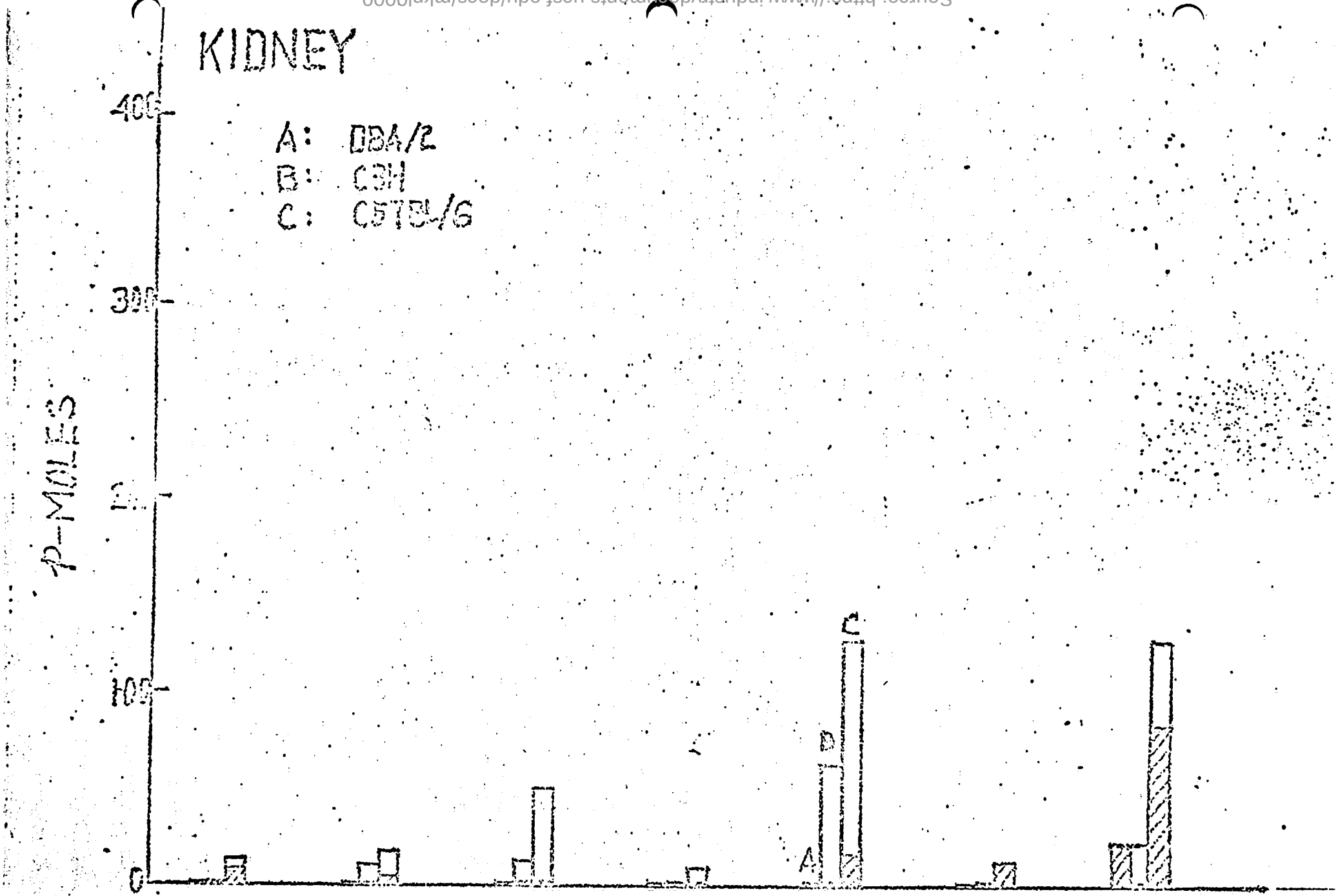

Fig. 6

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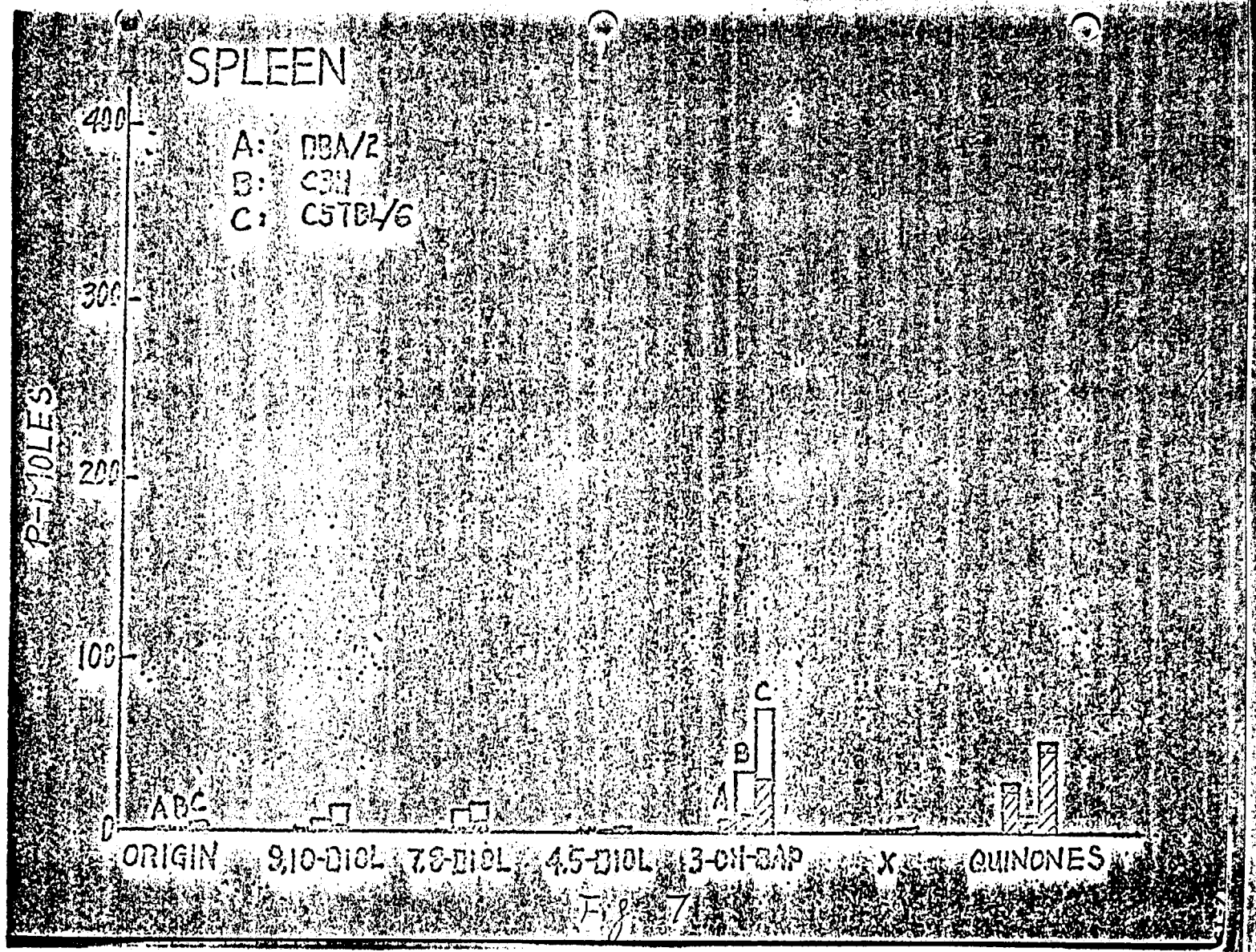

Fig. 7

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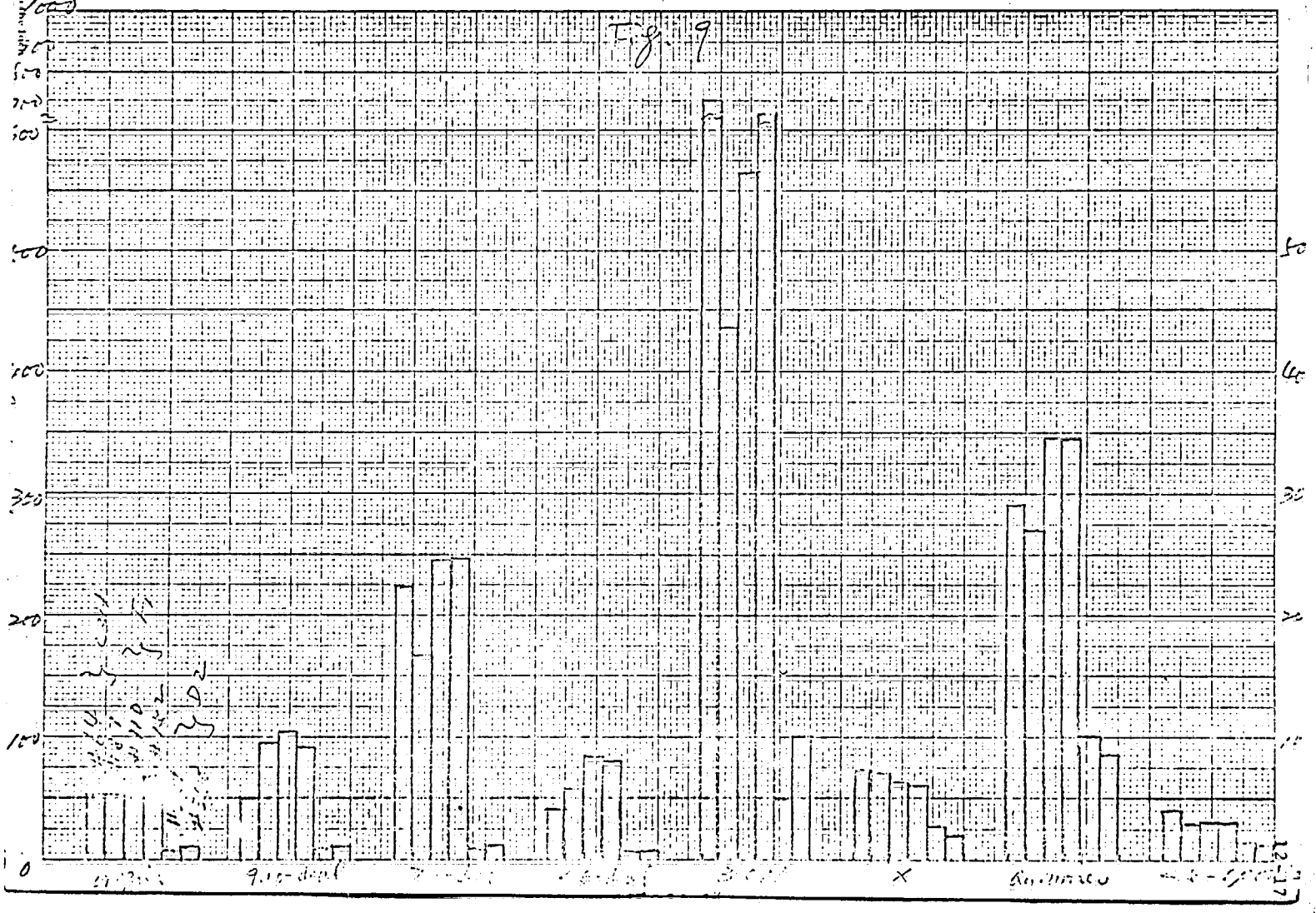

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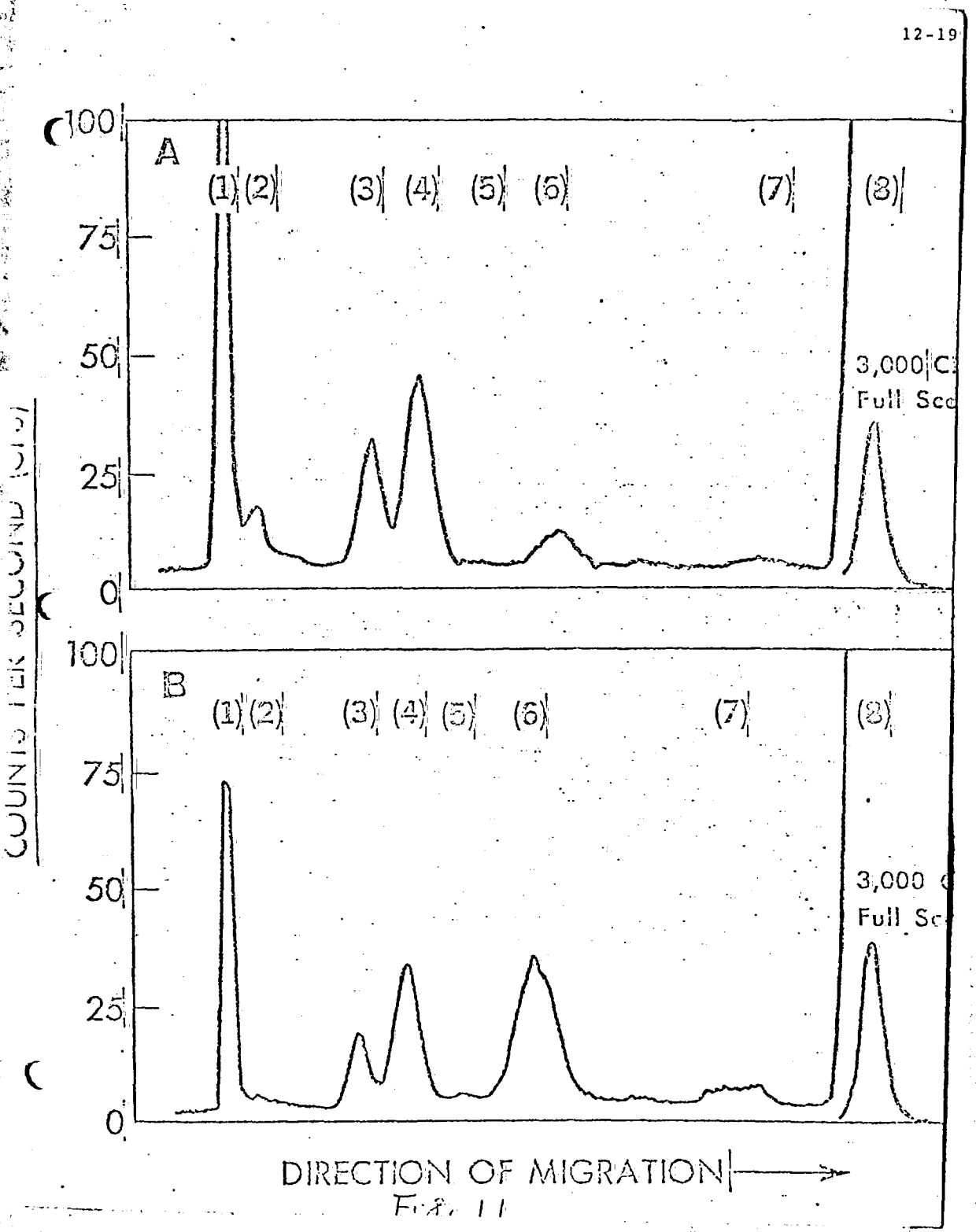

Fig. 11

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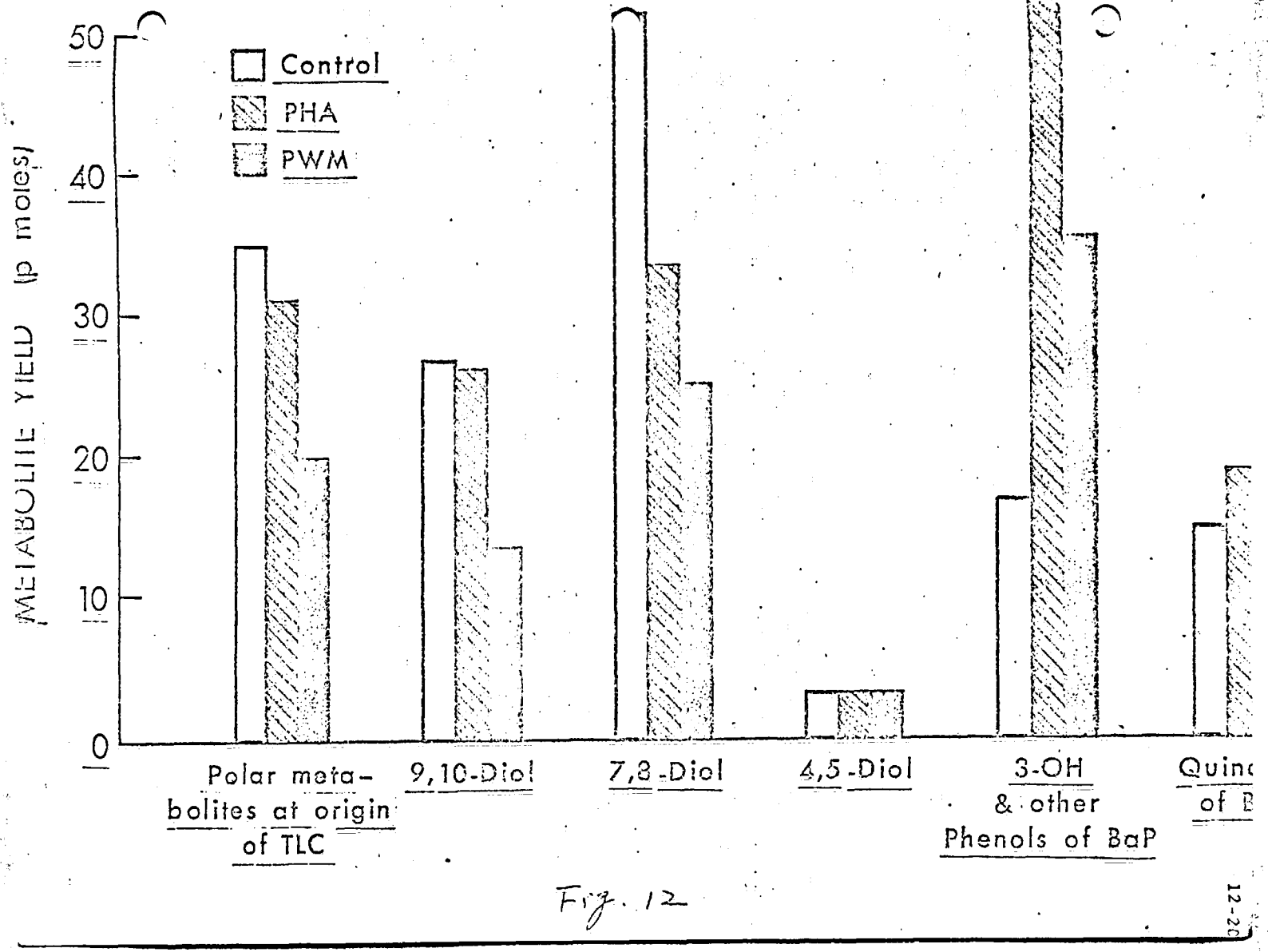

Fig. 12

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#1031-WATTENBERG

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

June 10, 1975

Grant Application No. 1031

CANCER

To: The committee comprising Drs. Gardner, Huebner and Meier

Subject: Lee W. Wattenberg, M.D., University of Minnesota Medical School, Minneapolis
New application No. 1031
"Inhibition of Carcinogenesis by Benzyl Isothiocyanate and Related Compounds"

History

This application was not handled as a case. On several occasions Dr. Wattenberg has acted as an outside reviewer for the CTR.

Request

Application No. 1031 requests $90,707 for the first year of a three year program.

Documents submitted (attached)

1. Application dated May 29, 1975 (18 pages including C.V. of Dr. Wattenberg)

2. Four publications and one manuscript

D.S.

DS:wg
Encl.

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Original #103

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUN 6 1975

Date: May 29, 1975

Application for Research Grant

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Lee W. Wattenberg, M.D. - Professor of Pathology

2. Institution & address:

University of Minnesota
Medical School
Department of Laboratory Medicine/Pathology
184M Jackson Hall
Minneapolis, MN 55455

3. Department(s) where research will be done or collaboration provided:

Laboratory Medicine & Pathology

4. Short title of study:

Inhibition of Carcinogenesis by Benzyl Isothiocyanate and Related Compounds.

5. Proposed starting date: 10-1-75

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

1. To determine conditions under which benzyl isothiocyanate and related compounds will inhibit neoplasia resulting from exposure to polycyclic aromatic hydrocarbons.

2. To determine the capacity of these compounds to inhibit neoplasia due to other classes of carcinogens.

3. To determine the mechanism(s) of inhibition .

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8. *Brief statement of working hypothesis:*

Experiments have shown that benzyl isothiocyanate will inhibit BP-induced neoplasia of the stomach and lung of the mouse and DMBA-induced mammary tumor formation in the rat. The mechanism(s) of this inhibition is not known nor have the conditions under which it occurs been defined. Inhibition may be due to direct interaction of benzyl isothiocyanate with reactive metabolites of the carcinogens. An alternate possibility is that the metabolism of the carcinogens have been changed by an effect of benzyl isothiocyanate on the enzymes carrying out these reactions. Both possibilities will be explored.

9. Details of experimental design and procedures (append extra pages as necessary)

- ATTACHED -

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Background Information. Studies which we have carried out recently have shown that benzyl isothiocyanate and benzyl thiocyanate have the capacity to inhibit neoplasia resulting from exposure to polycyclic aromatic hydrocarbon (PAH) carcinogens (1). These compounds are primarily constituents of cruciferous plants, but are found in several other plant families as well. Of the two compounds, benzyl isothiocyanate is more potent and more versatile as an inhibitor of chemical carcinogenesis than the corresponding thiocyanate, so that much of the initial work centers on this compound. Benzyl isothiocyanate will inhibit mammary tumor formation in the rat when administered by oral intubation or intraperitoneally four hours prior to administration of a carcinogenic dose of DMBA. If benzyl isothiocyanate is added to a diet containing benzo(a)pyrene (BP) it will inhibit the occurence of tumors of the forestomach and lung resulting from this carcinogen. Benzyl thiocyanate will inhibit DMBA-induced mammary tumor formation but thus far has not been found to be effective in suppressing formation of gastric or pulmonary tumors. Sodium thiocyanate is inactive as an inhibitor in all systems which have been studied. The mechanism(s) by which benzyl isothiocyanate inhibits neoplasia is not known. Of possible significance are biochemical studies which have shown that if this compound is added to a microsomal system in vitro, AHH activity is inhibited.

Effect of benzyl isothiocyanate on the nature and quantity of metabolites of BP formed by the microsomal mixed function oxidase system. The initial studies that we proposed carrying out will be directed at determining possible quantitative and qualitative differences in metabolites which occurs when BP is incubated with microsomes from lung, liver or squamous stomach of mice fed

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benzyl isothiocyanate as compared to corresponding controls. High pressure liquid chromatography (hplc) will be employed following recently described procedures (2,3). This technique results in a separation of a large number of metabolites, including phenols, diols, and quinones. Thin-layer chromatography using tritium-labelled BP will be employed as an adjunct to the work with hplc. In results with hplc presented thus far, the presence of 6-OH-BP and BP epoxides have not been reported. It is likely that these compounds are too reactive to survive the workup procedures and high temperature conditions (50°) of the hplc techniques used thus far. To trap these highly reactive species, we plan to employ low temperature extraction with the exclusion of light and oxygen and to use ambient temperature hplc. In addition, efforts will be made to form derivatives of the metabolites with various silylating agents. These silyl derivatives will be analyzed by glc and mass spectroscopy. An alternative possibility for studying 6-OH-BP is to employ the method of Ts'o et al (4,5). Depending upon the results obtained, additional analytical procedures or modifications of the procedures described will be employed.

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Effects of benzyl isothiocyanate on binding of BP metabolites to DNA. Current evidence suggests that the extent of macromolecular binding of carcinogen metabolites is a critical parameter for determining the neoplastic potential of the carcinogen under investigation. Accordingly, it would be of importance to determine to what extent the magnitude of DNA binding is altered by administration of benzyl isothiocyanate under conditions in which it inhibits chemical carcinogenesis. The initial studies of this type will be carried out employing an experimental model in which benzyl isothiocyanate is fed at a dose level resulting in inhibition of neoplasia. Microsomes from lung, liver, and

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small bowel will be isolated. These will be used for studying DNA binding of BP metabolites under conditions where the parent carcinogen is incubated in the presence of liver microsomes, NADPH, and DPN. ^{14}C-labelled BP will be employed. Following incubation, the DNA will be isolated employing a modification of the procedure used by Grover and Sims (6).

Studies of DNA binding with BP-7, 8-dihydrodiol. Recent work has shown that incubation of liver microsomes with BP-7,8-dihydrodiol, a metabolite of microsomal metabolism of BP, results in a high degree of binding to DNA (7). In addition, studies by Brooks et al indicate that the bound product has characteristics similar to that of the bound metabolites of BP which are formed in vivo (8). These data suggest that one of the possible pathways by which BP becomes bound to DNA entails basically two metabolic cycles. In the first, BP is converted by microsomes to the 7,8-dihydrodiol. Subsequently, this metabolite is metabolized again by the microsomal mixed function oxidase system and this time is converted to BP-7,8-dihydrodiol,9,10-epoxide. This latter compound is highly reactive and binds to DNA. In the work proposed, we would like to determine whether the microsomes from benzyl isothiocyanate fed mice as compared to control mice differ in their capacity to convert the BP-7,8-dihydrodiol to the metabolite which binds to DNA. Reaction conditions comparable to those used for the DNA binding studies in which BP is employed will be utilized. Parallel studies will be run employing both BP and BP-7,8-dihydrodiol.

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Studies of epoxide metabolism. Epoxides are formed as intermediate products of BP metabolism and can react with cellular macromolecules such as DNA. Epoxide hydrase is a microsomal enzyme which converts epoxides to dihydrodiols. This constitutes an important step in the metabolism of BP as well as other polycyclic hydrocarbons. Accordingly, we propose to determine the effects of benyzl isothiocyanate administration on the activity of this enzyme. The procedure

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employed will be based on that of Oesch et al (9). Tritiated styrene oxide or benzo(a)pyrene-4,5-oxide will be used as substrates. Another pathway of epoxide metabolism entails conjugation with glutathione (glutathione S-epoxide transferase). This conjugation will be studied employing the procedure of Booth and Sims (10).

Studies of formation of BP conjugates. In addition to the experiments described above, procedures will be carried out to study glucuronide and sulfate conjugation of BP in benzyl isothiocyanate fed and control animals. Cell fractions from liver or forestomach of control and benzyl isothiocyanate fed mice will be incubated with BP or BP metabolites and added uridine diphosphate-D-glucuronic acid (UDPGA) or 3-phosphoadenosine-5'-phosphosulfate (PAPS). High pressure liquid chromatography in an ion-exchange mode will be used to study water-soluable conjugates from the above reactions. The metabolites of BP which will be used as substrates initially are 3-OH-BP, 6-OH-BP, and BP-7,8-dihydrodiol. The 3-OH-BP is a relatively stable metabolite which will be useful as a prototype of conjugation of a monophenol derivative. The 6-OH-BP is an unstable compound and reaction conditions for its use may be difficult to work out. However, it is an important compound being a possible proximate carcinogen, so that its rate of conjugation could determine the amound available for macromolecular binding. Accordingly, efforts will be made to establish a procedure which can quantitatively determine its conjugation. BP-7,8-dihydrodiol,9,10-oxide reacts with macromolecules. If BP-7,8-dihydrodiol is conjugated, the epoxidation would be prevented. Thus, this substrate is important to study because of its potential biological effects. It also represents a prototype of a dihydrodiol metabolite of BP. In later studies, other metabolites of BP will be employed as substrates. After the methodology has been established for identifying and quantitating the conjugates, studies of their biliary and urinary excretion in

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control and BHA fed mice given ^{14}C-BP will be attempted.

Studies of the reactions between benzyl isothiocyanate and metabolite(s) of BP in a microsomal system. Inhibition of polycyclic hydrocarbon induced neoplasia by benzyl isothiocyanate may possibly be the result of a reaction between a highly reactive metabolite or metabolites of BP and benzyl isothiocyanate. In essence, what would be occurring is that the isothiocyanate scavenges the reactive form of the carcinogen metabolite before it has a chance to bind to macromolecules. If this mechanism exists, it might be possible to demonstrate a reaction product between benzyl isothiocyanate and BP metabolite(s). Current evidence indicates that there are two principle types of highly reactive metabolites of BP which can bind to macromolecules. The first consists of a group of arene oxides and the second, positively charged radicals (5,11-14). We propose carrying out three types of experiments. The first will be studies of the reactions of reactive metabolites of BP belonging to these two groups with benzyl isothiocyanate in simple solutions. The second will be an effort at demonstrating the presence of reaction product(s) after incubation of BP and benzyl isothiocyanate in a microsomal system and the final study will be an effort demonstrating the occurance of reaction product(s) in vivo. For the initial studies of the interactions of active metabolites of BP and benzyl isothiocyanate in solution we propose to employ three BP derivatives. The first two are BP-4,5-oxide and BP-7,8-dihydrodiol,9,10-oxide. A great deal of evidence has implicated arene oxides as proximate carcinogens of BP (8,11,12). The evidence for BP-7,8-dihydrodiol,9,10-oxide being a proximate carcinogen has been presented previously. The third is the 6-oxo-BP radical which forms from 6-OH-BP (4,5). This is a highly reactive radical which binds to macromolecules. Studies of Ts'o and colleagues have shown that 6-OH-BP comprises about

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15% of the total metabolites formed on incubation of BP with liver microsomes (4). Thus from both qualitative and quantitative aspects, this compound is of considerable interest. The active metabolites of BP will be allowed to react with benzyl isothiocyanate both in aqueous and organic solvent systems and the products of these reactions isolated. Their structures and physical and chemical properties will be studied, including behavior in procedures such as high pressure liquid chromatography and thin layer chromatography which will be used in subsequent identification procedures in complex reaction mixtures.

The second group of studies will consist of incubations of ^{3}H-BP and ^{14}C benzyl isothiocyanate in a liver microsomal system. Possible interaction products along with the metabolites and starting materials will be extracted into organic solvents from the incubation mixture. High pressure liquid chromatography and thin layer chromatography will be used to separate possible interaction products from the rest of the metabolites and would be identified by the presence of both ^{3}H and ^{14}C radioactivities in appropriate ratios. The finding of such entities would be followed by efforts at large scale isolation and structural elucidation of reaction products. In order for this to be accomplished, isolation of workable quantities of pure materials are necessary. With such materials, physical data such as melting points; elemental analysis; spectral data including infrared, ultraviolet, and fluorescence; nuclear magnetic resonance; and mass spectroscopic analysis will be obtained for determination of chemical structure. These data will be compared with those of the chemically synthesized reference compounds. If a reaction product or products can be identified in an in vitro system, further work will be carried out in an effort to demonstrate the comparable material in vivo. This will entail administration of ^{3}H-BP and ^{14}C benzyl isothiocyanate to mice in subsequent attempts to establish the presence of the reaction product in tissues.

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Studies of conditions under which benzyl isothiocyanate will inhibit BP carcinogenesis. Work will be carried out with four animal model systems to determine conditions under which benzyl isothiocyanate will inhibit BP carcinogenesis. From these studies it should be possible to obtain information as to the effects of various routes of administration of inhibitor and carcinogen and also basic information on animal models most effective for additional studies. Such studies will include dose-response, those involving other compounds to be investigated for their inhibiting properties, and also work with additional carcinogens. The first animal model will entail the the use of Ha/ICR mice (15). These animals were employed in work already carried out which showed that addition of benzyl isothiocyanate to a diet containing BP will inhibit the formation of gastric tumors as well as pulmonary tumors. We propose to carry out two further studies with this animal model. In the first, the carcinogen will be given by oral intubation rather then added directly to the diet. This has the advantage of providing precise information as to the amount of carcinogen consumed. In a second modification carcinogen and benzyl isothiocyanate will both be given by oral intubation. The second set of studies will entail the use of A/HeJ mice. These animals have a high susceptibility to pulmonary neoplasia. A great deal is known about the response of this animal model to various carcinogens and factors which alter carcinogenicity. Thus it is a versatile system for studying inhibition of neoplasia. We propose carrying out a study in which the carcinogen is given by oral intubation and the benzyl isothiocyanate in the diet, a second study in which both carcinogen and benzyl isothiocyanate are given by oral intubation, a third study in which the carcinogen is given by oral intubation and the benzyl isothiocyanate is given intraperitoneally, a fourth study in which the carcinogen is given intraperitoneally and the benzyl isothiocyanate by oral intubation, and finally two studies in which the carcinogen

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is given intravenously. In one the benzyl isothiocyanate will be given by oral intubation and in the second it will be given intraperitoneally. In the third animal model, BP will be administered by the intratracheal route employing a technique comparable to that described by Ho and Furst (16). Benzyl isothiocyanate will be given by oral intubation, i.v. or i.p. The fourth model that will be employed will be that of DMBA-induced mammary tumor formation. Previous work has shown that benzyl isothiocyanate given either i.p. or by oral intubation prior to administration of DMBA by oral intubation, will result in inhibition of mammary tumor formation. We propose to carry out modifications of this procedure in which the carcinogen is given intravenously instead of by oral intubation and the benzyl isothiocyanate either by oral intubation or i.p..

Studies of inhibition by benzyl isothiocyanate of carcinogenesis from carcinogens other than polycyclic aromatic hydrocarbons. In these studies we propose initially to use A/HeJ mice employing conditions found most effective for inhibiting pulmonary adenoma formation in the studies described above. Work will be carried out with several carcinogens. These include diethylnitrosamine, urethane, uracil mustard and 4-nitroquinoline-N-oxide. These studies will indicate whether inhibition by benzyl isothiocyanate occurs with a variety of carcinogens other than polycyclic hydrocarbons and including one carcinogen; namely, uracil mustard, which is a direct alkylating agent. Depending on the results obtained, considerations will be given to work with other test systems and carcinogens. In addition significant inhibitors identified in the final section of this proposal will be studied against a variety of carcinogens in a manner comparable to benzyl isothiocyanate.

Dose-response studies. On the basis of the findings of the initial portion of the animal work with the four animal model systems, dose-response studies will be carried out to

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determine the capacity of various dose levels of benzyl isothiocyanate to inhibit BP carcinogenesis in several experimental systems. When these data become available consideration will be given to carrying out further work involving other carcinogens.

Studies of the relationships between chemical structure and carcinogen-inhibiting activity of compounds related to benzyl isothiocyanate. We propose to determine the inhibitory capacities of several other isothiocyanates against model systems described above using conditions which appear to be the most appropriate for comparative studies. The compounds to be employed will be of two types. The first are compounds which occur naturally and accordingly could have some impact on response to chemical carcinogens. These include phenylethyl isothiocyanate, p-hydroxybenzyl isothiocyanate and allyl isothiocyanate. In addition to these naturally occurring compounds, we propose to study several compounds related to benzyl isothiocyanate in which there is a varying ease of hydrolysis of the isothiocyanate group. One of the possibilities is that when the benzyl isothiocyanate enters the cell, the isothiocyanate group is hydrolyzed off and that this is the effective component. A second possibility is that the intact molecule is required for inhibitory activity. In order to investigate this question we propose to employ phenyl isothiocyanate, a compound in which the isothiocyanate group will not hydrolyze and also a series of para-substituted derivatives of benzyl isothiocyanate with differing electron withdrawing or donating activity i.e. CH_3O, CH_3S, CH_3, Cl and NO_2. An additional compound related to benzyl isothiocyanate that we propose to study is benzyl nitrile. Nitriles are also naturally occurring components of plants. Studies which have been carried out have shown that some nitriles have the capacity to inhibit carcinogenesis resulting from administration of nitrosamines. Accordingly benzyl nitrile would be of interest to study under comparable conditions to those

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employed for benzyl isothiocyanate. Depending on the results obtained with the above experiments additional compounds will be studied which have the potential of providing further information on inhibitory capacities of naturally occurring compounds, structural characteristics giving maximum inhibition and also those possibly giving insight into the mechanism of inhibition.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

General Bibliography

1. Wattenberg, L.W.: J. Natl. Cancer Inst. 52:1583-1587, 1974.

2. Selkirk, J.K., Croy, R.J., Roller P.P., and Gelboin, H.: Cancer Res 34:3474, 1974.

3. Holder, G., Yagi, H., Dansette, P., Jerina, D.M., Levin, W., Lau, A.Y., and Conney, A.H.: Proc. Nat. Acad. of Sci. 71:4356, 1974.

4. Lesko, S., Caspary, W., Lorentzen, R., and Ts'o, P.: National Meeting of the Biophysical Society, February-1974 (abstract).

5. Ts'o, P., Caspary, W., Cohen, B., Leavitt, J., Lesko, S., Lorentzen, R., and Schectman, L.: In Chemical Carcinogenesis Part A - P Ts'o and J. DiPaolo eds. Marcel Dekker, N.Y., 1974, pp. 113.

6. Grover, P.L., Sims, P.: Biochem J 110:159-160, 1968.

7. Borgen, A., Darvey, H., Castagnoli, N., Crocker, T.T., Rasmussen, R.E., and Wang, I.Y.: J. Medicinal Chem 16:502, 1973.

8. King, H., Alexandrov, K., Thompson, M.H., Osborne, M., and Brookes, P.: Proc. Am. Assoc. for Cancer Res. 16: 197, 1975.

9. Oesch, F., Jerina, D.M., and Daly, J.: Biochemica Biophys. Acta. 227:685, 1971

10. Booth, J., and Sims, P.: Biochem. Pharm. 24:2547, 1974.

11. Huberman, E., Kuroki, T., Marquardt, H., Selkirk, J.K., Heidelberger, C., Grover, P.L., and Sims, P.: Cancer Res. 32:1391-1396, 1972.

12. Jerina, D.M., and Daly, J.W.: Science 185:573-581, 1974.

13. Nagata, C., Inomata, M., Kodama, M., and Tagashira, Y.: Gann 59:289-298, 1968.

14. Cavalieri, E., and Calvin, M.: Proc. Nat. Acad. Sci. U.S. 68:1251-1253, 1971.

15. Wattenberg, L.W.: J. Natl. Cancer Inst. 48:1425-1430, 1972.

16. Ho, W., and Furst, A.: Oncology 27:385, 1973.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Three large laboratories and ample animal quarters under the direction of the principal investigator are available for use in this project. The laboratories are equipped with facilities for carrying out work in biochemistry and organic chemistry. They contain the following equipment: Liquid chromatograph (Waters Associates), Packard Tri-Carb liquid scintillation spectrometer 2425, Packard Model 420 gas chromatograph, Gilson linear fraction collector, Aminco-Bowman spectrofluorophotometer, Beckman Model J-21B centrifuge, Servall refrigerated centrifuges, balances, pH meters, and other equipment for biochemical work. A walk-in cold room, refrigerators and deep freeze (-50°C) units are present. Equipment for carrying out organic syntheses and purification of compounds is present. Equipment for carrying out routine and special histologic staining techniques is in the laboratories. In addition to our own equipment, the Pathology Department has shared equipment including a Beckman LS-250 Liquid scintillation counter. Specialized equipment for use in determining chemical structure such as nuclear magnetic resonance spectrographs and mass spectrometers are available for our use in the Department of Chemistry.

11. Additional facilities required:

None.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

BIOGRAPHICAL SKETCH

NAME Lee W. Wattenberg, M.D. TITLE Professor of Pathology

BIRTH DATE REDACTED PLACE OF BIRTH REDACTED

NATIONALITY REDACTED

EDUCATION

College of the City of New York, New York	B.S.	1941	Premedicine
University of Minnesota, Minneapolis	B.M.	1949	Medicine
University of Minnesota, Minneapolis	M.D.	1950	Medicine

HONORS AND PROFESSIONAL ASSOCIATIONS

Alpha Omega Alpha
Sigma Xi
Borden Award Undergrad. Medicine Research
Lederle Medical Faculty Award 1956-59
President, American Histochemical Society 1966-67
Associate Editor, Cancer Research

RESEARCH AND PROFESSIONAL EXPERIENCE

Professor of Pathology - University of Minnesota	1966 - present
Associate Professor of Pathology - University of Minnesota	1959 - 1966
Visiting Scientist - Chester Beatty Research Institute	1963 - 1964
Assistant Professor of Pathology - University of Minnesota	1957 - 1959
Instructor, Department of Pathology - University of Minnesota	1955 - 1957
Medical Fellow - Department of Pathology - University of Minnesota	1954 - 1955
U.S. Army - 1st Lt. - (Dr. Joseph Smadel) Virus and Rickettsial Disease Department - Walter Reed Medical Center	1952 - 1954
Research Fellow, American Cancer Society - University of Minnesota Departments of Biochemistry and Pathology	1950 - 1952
Internship, Department of Medicine - University of Minnesota	1949 - 1950
Jr. Biologist, Manhattan Project, Medical Research Group	1944 - 1946

SELECTED BIBLIOGRAPHY

1. Alterations in Microsomal Metabolism of Benzo(a)pyrene in Mice by Butylated Hydroxyanisole. Jennine L. Speier and Lee Wattenberg. J. Natl. Cancer Inst. 54 1975 - (Preprint enclosed).

2. Aryl Hydrocarbon Hydroxylase Induction in Rat Tissues by Naturally Occurring Indoles of Cruciferous Plants. William D. Loub, Lee W. Wattenberg and David W. Davis. J.Natl. Cancer Inst. 54, 1975 (Reprint enclosed).

3. Inhibition of Carcinogenic and Toxic Effects of Polycyclic Hydrocarbons by Several Sulfur-Containing Compounds. Lee W. Wattenberg. J. Natl. Cancer Inst. 52:1583-1587, 1974 - (Reprint enclosed).

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4. Inhibition of Chemical Carcinogen Induced Pulmonary Neoplasia by Butylated Hydroxyanisole. Lee W. Wattenberg. J. Natl. Cancer Inst. 50:1541-1544, 1973 (Reprint enclosed).

5. Inhibition of Carcinogenic and Toxic Effects of Polycyclic Hydrocarbons by Phenolic Antioxidants and Ethoxyquin. Lee W. Wattenberg. J. Nat. Cancer Inst. 48:1425-1430, 1972 - (Reprint enclosed).

NAME Peter Borchert

TITLE Senior Scientist

BIRTHDATE REDACTED

PLACE OF BIRTH REDACTED

NATIONALITY REDACTED

EDUCATION

TH-Darmstadt College - Germany (Graduate School) Universität Heidelberg - Heidelberg, Germany	B.S	1964	Chemistry
	M.S.	1968	Chemistry
University of Wisconsin - Madison, Wisconsin	Ph.D.	1972	Oncology

RESEARCH AND PROFESSIONAL EXPERIENCE

University of Minnesota - Department of Laboratory Medicine & Pathology - Senior Scientist	1973 - present
McArdle Laboratory - Madison, Wisconsin - Post-doctoral Fellow	12/1972 - 9/1973
University of Wisconsin - McArdle Laboratory - Madison, Wisconsin Graduate Student	6/1968 - 11/1972
Universität of Heidelberg - 69 Heidelberg, Germany Graduate Student	10/1964 - 5/1968

SELECTED BIBLIOGRAPHY

1. The Metabolism of the Naturally-occurring Hepatocarcinogen Safrole to 1'-Hydroxysafrole and the Electrophilic Reactivity of 1'-Acetoxysafrole. Borchert, P., Wislocki, P.G., Miller, J.A., & Miller, E.C. Cancer Research 33: 575-589, (1973).

2. 1'-Hydroxysafrole: A Proximate Carcinogenic Metabolite of Safrole in the Rat and Mouse. Borchert, P., Miller, J.A., Miller, E.C., and Shires, T.K. Cancer Research 33:590-600, (1973).

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Dr. Lee Wattenberg	15	----
Dr. Peter Borchert (Senior Scientist)	50	REDACTED
Technical		
Allan Fladmoe (Junior Scientist - Chemist)	100	
Junior Scientist - with credentials for animal work (to recruit)	100	REDACTED
Animal Caretaker (to recruit)	100	9,112
Fringe Benefits (17%)		7,827
Sub-Total for A		53,867
B. Consumable supplies (by major categories)		
Purchase and care of experimental animals		6,000
Purified diets		2,000
Chemicals		4,000
Glassware and misc. small items of equipment		2,500
Isotopically labelled compounds*		7,000
*(^{14}C-benzyl isothiocyanate, ^{14}C-benzo(a)pyrene, ^{3}H-benzo(a)pyrene)		
Sub Total for B		21,500
C. Other expenses (itemize)		
Travel - to scientific meetings		900
Sub-Total for C		900
Running Total of A + B + C		76,267
D. Permanent equipment (itemize)		
Fraction collector holding vials for scintillation counter (Gilson Company)		3,000
Sub-Total for D		3,000
E. Indirect costs (15% of A+B+C)		11,440
Total request		87,707

15. Estimated future requirements.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2 (increased by 10% over previous yr)	59,507	21,500	800	0	12,271	94,078
Year 3 (increased by 10% over previous yr)	65,735	22,500	800	0	13,355	102,390

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16. Other sources of financial support

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Inhibition of Chemical Carcinogenesis by Anti-oxidants	USPHS CA-14146	90,000	2-1-73 to 2-1-76
Inhibition of Neoplasia of the Large Bowel	USPHS CA-15638	143,300	1-1-74 to 12-31-76
Studies of Polycyclic Hydrocarbon Metabolism in the Respiratory Tract	USPHS CA-33364 Contract	177,200	12-23-74 to 12-22-76
Histochemical & Quantitative Studies of Neoplasia	USPHS CA-09599	248,755	9-1-70 to 8-31-75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Inhibition of Chemical Carcinogenesis by Anti-oxidants	USPHS CA-14146 (renewal application)	503,402	2-1-76 to 1-31-81
Histochemical and Quantitative Studies of Neoplasia	USPHS CA-09599 (renewal application	473,780	9-1-75 to 8-31-80

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Lee W. Wattenberg

Signature Lee W. Wattenberg Date 5/29/75

Telephone 612 373-2808
Area Code Number Extension

Checks payable to

University of Minnesota

Mailing address for checks Mr. Clinton Johnson
Assistant Vice President
University of Minnesota
302 Morrill Hall
Minneapolis, MN 55455

Responsible officer of institution

Typed Name C. T. Johnson

Title Assistant Vice President

Signature C. T. Johnson Date June 4, 1975

Telephone 612 373-2058
Area Code Number Extension

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#1032 - STEIN

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 16, 1975

Grant Application No. 1032

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Gardner, and Sommers

Subject: Olga Stein, M.D., Hebrew University-Hadassah Medical School, Jerusalem
New application No. 1032
"Endothelial Cell Structure and Function"

History

An informal inquiry was handled as Case No. 330, and application was encouraged.

Request

Application No. 1032 requests $29,900 for the first year of a three year program.

Documents submitted

1. Application dated June 5, 1975 (7 pages, including C.V. of Dr. Stein).

2. Five reprints.

Comment

Signature of responsible officer of institution is missing from page 5, but this will be received prior to the SAB meeting.

D.S.

DS:wg
Encl.

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H 1032

The Council For Tobacco Research–U.S.A., Inc.

110 East 59th Street
New York, N. Y. 10022
(212) 421-8885

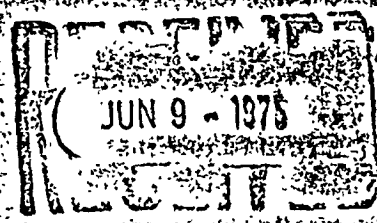
Application for Research Grant

(Use extra pages as needed)

Date: June 5, 1975

1. Principal Investigator (give title and degrees):

Olga Stein, M.D., Professor of Experimental Medicine



2. Institution & address:

Hebrew University-Hadassah Medical School
Jerusalem, Israel POB 1172

3. Department(s) where research will be done or collaboration provided:

Department of Experimental Medicine and Cancer Research and Department of Medicine

4. Short title of study:

Endothelial cell structure and function

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The specific aims of this research are related to the study of the glycoprotein residue which constitute a part of the endothelial cell surface coat. These glycoprotein residue will be identified with the help of specific lectins and attempts will be made to estimate their amount. The rate of renewal of the surface material glycoprotein under normal conditions will be studied and the effect of agents which modify various secretory processes will be determined. It is proposed also to determine the relation of surface material glycoproteins to the lipoprotein lipase that is active at the endothelial cell surface.

The information obtained under controlled conditions with normal tissue will serve as a basis for investigations in which the effect of various pollutants on the formation and maintenance of the endothelial surface coat can be studied.

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8. Brief statement of working hypothesis:

The endothelial cell surface can be visualized with ruthenium red, which apparently stains polysaccharide residues. The amount of the surface coat could vary under different conditions and hence a quantitation of the material will be attempted. The mode of formation of the material has not been elucidated and its rate of formation might be affected by agents which modify transport of secretory products or membrane precursors. The endothelial cell surface has been considered as the site of action of lipoprotein lipase, and hence a possible relation between this enzyme and the surface glycoproteins will be sought.

9. Details of experimental design and procedures (append extra pages as necessary)

For the identification of glycoprotein residues in the surface of endothelial cells several lectins will be used which bind to specific sugar residues. Concanavalin A, wheat germ agglutinin phytohaemagglutinin and ricin for α-D-mannose, α-D-glucose, α-D-fructose, (Con A) N-acetyl glucosamine, N-acetylgalactosamine and d-galactose, respectively. In order to render them identifiable by electron microscopy the lectins will be complexed to peroxidase (1) or ferritin (2) and purified to remove unbound markers. Alternately the lectin will be visualized after it had been bound to the cell surface by reacting it with either horseradish peroxidase followed by the demonstration of enzyme activity or with hemocyanin, which binds specifically to ConA residues (3) and can be identified in shadow cast replicas. The lectins will be radioiodinated with I^{125} and purified from Sephadex G 50 columns (3).

The tissues to be studied will include the aorta, coronary arteries and myocardial capillaries of rats and mice and after the establishment of the techniques the most relevant experiments will be repeated in rabbits. In order to preserve the intactness of the endothelium the tissues will be studied after in vivo fixation with 1% paraformaldehyde, a procedure which was shown not to affect the quantity or specificity of binding of concanavalin A (3). Following exposure to the lectin its visualization will be carried out on tissues prepared for electron microscopy. When peroxidase will be used to reveal the presence of lectins, sections will be prepared with a Smith-Farquhar tissue sectioner and peroxidase activity will be visualized with the Karnovsky procedure. Following post-fixation with osmium tetroxide, dehydration and embedding, ultrathin sections will be examined with the Philips EM 300 electron microscope. To study the coronary arteries and heart capillaries use will be made of coronary perfusion to introduce the lectins into the vascular lumen. Preliminary experiments have shown that binding of concavalin A to the endothelial cell surface introduced by perfusion of coronary circulation can be demonstrated at the surface of heart capillaries both by radioautography and peroxidase activity. For the validation of the specificity of binding, appropriate sugar derivatives specific for each lectin will be used to prevent binding or to dissociate the bound lectin. In order to minimize endocytosis when unfixed hearts will be studied the perfusion will be carried out at 10°-18°C. The quantitation of the binding sites will be attempted with the help of

A. radioautography at the light microscope or electron microscope level, after exposure of sections to radioautographic emulsion
B. counting of ferritin on properly enlarged electron micrographs
C. in the case of the aorta use will be made also of hemocyanin. After exposure to hemocyanin the tissue will be fixed and dehydrated in ethanols and dried, preferably with critical point drying. Replicas will be prepared, cleaned and examined under the electron microscope.

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9. continued

To study renewal of the surface coat glycoproteins use will be made of ^{3}H-fucose, which is known to be incorporated into glycoproteins (4). The rats or mice will be injected with the labeled precursor and tissues prepared for radioautography at 5-120 min time intervals. The distribution of the radioautographic grains will be determined over the cell body and the cell surface. In order to learn about the effect of agents, which modify the transport of secretory products, on the rate of renewal of surface glycoproteins, the animals will be pretreated with colchicine or vinblastin for 1-4 h prior to labeling with ^{3}H-fucose.

To study the relation between lipoprotein lipase and the surface glycoproteins use will be made of the various lectins. In order to determine whether the sugar residues of the membrane bound enzyme are exposed, hearts will be perfused with radioiodinated lectins and then lipoprotein lipase will be released into the perfusat by introduction of heparin (5). In preliminary experiments it has been learned that concanavalin A does not interfere with the measurement of enzyme activity.

1. N.K. Gonatas and S. Avrameas. J. Cell Biol. 59:436, 1973.
2. G.L. Nicolson and S.J. Singer. Proc. Natl. Acad. Sci. 68:942, 1971.
3. S.B. Smith and J.P. Revel. Develop. Biol. 27:439, 1972.
J.Z. Rosenblith, T.E. Ukena, H.H. Yin, R.D. Berlin and M.J. Karnovsky, Proc. Nat. Acad. Sci. 70:1625, 1973.
4. J. Bennet, C.P. Leblond and H. Haddad. J. Cell Biol. 60:258, 1974.
5. E. Atkin and H.C. Meng. Diabetes 21:149, 1972.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The work on the project will be carried out in the laboratory for electron microscopy of the department of experimental medicine and cancer research and in the lipid research laboratory of the department of medicine. Both laboratories are located in the same building and have adequate facilities for both the morphological and biochemical studies. These include electron microscope facilities, ultramicrotomes, dark rooms, scintillation counters, cold rooms and animal rooms.

11. Additional facilities required:

Not applicable

10035402/27

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

12. Biographical sketches of investigator(s)

Olga Stein, principal investigator

1952 M.D. from Hebrew University Hadassah Medical School, Jerusalem
1953-1958 Department of Pathology, Hebrew University Hadassah Medical School
1958-present Department of Experimental Medicine and Cancer Research Med. School, Jerusalem
1960-1961 and 1967-1968 Visiting Scientist, The Rockefeller University, New York
1969 Associate Professor, Experimental Medicine, Hebrew University
1974 Professor of Experimental Medicine, Hebrew University
1975 Visiting Professor, University of California, San Diego

Tova Chajek, coinvestigator

1971 M.D. from Hebrew University Hadassah Medical School, Jerusalem
1971-present Department of Medicine B, Hadassah University Hospital, Jerusalem, and in Lipid Research Laboratory
1972 Instructor in Medicine
1974 Lecturer in Medicine

Yechezkiel Stein, coinvestigator

1952 M.D. from Hebrew University Hadassah Medical School, Jerusalem
1953-present Department of Medicine B, Hadassah University Hospital, Jerusalem
1963 Associate Professor of Medicine
1965 Head of Lipid Research Laboratory
1960-1961 and 1967-1968 Visiting Scientist, Rockefeller University, New York
1969 Professor and Head of Department of Medicine
1975 Visiting Professor, Rockefeller University, N.Y. and University of California, San Diego

13. (1) CIBA Found. Symp. 12:165, 1973
(2) J. Cell Biol. 62:90, 1974
(3) Biochim. Biophys. Acta 360:205, 1974
(4) Biochim. Biophys. Acta 380:106, 1975
(5) Biochim. Biophys. Acta 380:127, 1975

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

R: REDACTED MATERIAL

14. First year budget.

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
O. Stein, M.D.	30%	-
T. Chajek, M.D.	25%	-
Y. Stein, M.D.	10%	-
Technical		
Technician for electron microscopy, M.Sc.grade	100%	REDACTED
Technician for biochemical study, M.Sc. grade	100%	-

*includes social benefits

Sub-Total for A REDACTED

B. Consumable supplies (by major categories)

Lectins (Con A, WGA, Ricin)	1,000
Chemicals, including osmic acid	2,000
Glassware	1,000
Isotopes (^{3}H Fucose, ^{125}I)	1,000
Electron microscopy supplies	1,000

Sub-Total for B 6,000

C. Other expenses (itemize)

Animals + upkeep	2,000

Sub-Total for C 2,000

Running Total of A + B + C 26,000

D. Permanent equipment (itemize)

None

Sub-Total for D

E. Indirect costs (16% of A+B+C) E 3,900

15. Estimated future requirements. Total request 29,900

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	6,500	2,500	4,200		33,350
Year 3	R	7,000	3,000	4,800		36,800

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Lipid metabolism by smooth muscle cells in culture	U.S.-Israel Binational Science Foundation (Grant to Y. Stein)	$15,000	8/1/75-7/31/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Olga Stein

Signature Olga Stein Date 6/14/75

Telephone 212 (Area Code) 4111 (Number) 475 (Extension)

Checks payable to

Mailing address for checks

Responsible officer of institution

Typed Name

Title

Signature Date

Telephone (Area Code) (Number) (Extension)

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#1037 - FRIBERG

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 26, 1975

Grant application No. 1037

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Jacobson, Lynch and Sommers

Subject: Lars Friberg, M.D., The Karolinska Institute, Stockholm
New application No. 1037
"Causes of Death in Relation to Smoking Habits and Other Behavorial and Environmental Factors. A study on the Swedish Twin Registry".

History

Dr. Friberg has been a grantee of CTR since November 1972, with an initial award of $3,000 for exploratory studies on the twin registry. Grant No. 892 (in the amount of $40,095 for the two year period January 1, 1973 to December 31, 1974) was awarded for the study entitled "Epidemiological Studies on the Swedish Twin Registry". A continuation grant (No. 892A) was awarded by the SAB in March 1975 (in the amount of $43,923 for the two year period July 1, 1975 through June 30, 1977) to support "Epidemiological Studies on the New Swedish Twin Registry".

The present new application (No. 1037) is a request to continue mortality follow-up studies previously supported by the American Medical Association Education and Research Foundation, at a level of $133,000 for a five year period ending January 31, 1976.

Request

Application No. 1037 requests $32,919 for the first year of a three year project, to begin February 1, 1976.

Documents submitted (attached)

1. Application dated June 17, 1975 (11 pages including enclosures 2 and 3).
2. One reprint (Enclosure 1).
3. Updated reports (Enclosures 4 and 5).

D.S.

DS:WG
Encl.

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#1037

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

JUN 23 1975
Date:

June 17, 1975

Application for Research Grant
(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Professor Lars Friberg, M.D., Co-investigator Docent Rune Cederlöf Ph.D.

2. Institution & address:

The Karolinska Institute
Department of Environmental Hygiene
S-104 01 Stockholm, Sweden

3. Department(s) where research will be done or collaboration provided:

Department of Environmental Hygiene

4. Short title of study: Causes of death in relation to smoking habits and other behavioral and environmental factors. A study on the Swedish twin registry.

5. Proposed starting date: February 1, 1975 ? 1976?

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: The Swedish twin registry, covering a total of about 11,000 twin pairs of the same sex, has been in operation since 1961. The main line of research has been to study gross and cause specific mortality against the background of smoking habits, alcohol problems and other smoking associated variables in twins. Results were reported in 1973 in the Archives of Environmental Health (enclosure 1). The report covered an 11 year study on 9,000 twin pairs, of same sex born 1901 to 1925.

In addition to intramural funds from the Karolinska Insitute and the Swedish National Environment Protection Board, during the years 1971 to 1975 the investigations were supported by a grant from the American Medical Association Education and Research Foundation. As the last mentioned agency is no longer in operation, the present application asks for financial support to continue the mortality follow-up study for the years 1976 through 1978.

Further details appear in enclosure 2.

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8. Brief statement of working hypothesis:

Smokers and nonsmokers can be assumed to differ in many other respects than smoking. These differences may be partly genetic and partly environmental. A direct comparison of the mortality outcome between smokers and nonsmokers in general cannot be made without due consideration to these factors. Research on smoking discordant twin pairs enhances the group comparability. Monozygotic twins offer complete genetic control, identity as to age and sex, and assumingly a far greater environmental similarity than smokers and nonsmokers in general. Fraternal twins constitute an ideal group for comparison.

Two extreme major hypotheses can be contrasted and put to test in the twin research design, namely

1. The observed statistical relationship between smoking and disease parameters is of a causal nature.
2. The observed statistical relationship between smoking and disease parameters is due to genetic and/or environmental factors correlated to smoking.

9. Details of experimental design and procedures (append extra pages as necessary)

See enclosure 2

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Ordinary office space and equipment

Computers: Nova 1220

Access to IBM 360/75, belonging to the University of Stockholm

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Known to the CTR

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See enclosure 3

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14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s) even if no salary requested)

	% time	Amount
Present salary through Dec. 1975		
Research ass. Ulla Lorich B.A.	100	13,857
Clerical ass.		4,000
Soc. security 33%		5,893
Expected annual increase 10%		2,375

1 $ = 3.89 Skr

Technical

Sub-Total for A 26,125

B. Consumable supplies (by major categories)

ok

Sub-Total for B

C. Other expenses (itemize)

Computer work 2,500

Sub-Total for C 2,500

Running Total of A + B + C 28,625

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C) E 4,294

Total request 32,919

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	28,737		2,500		4,686	35,923
Year 3	31,611		2,500		5,117	39,228

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
		$	
Causes of death in relation to smoking habits and other behavioral and environmental factors. A study on the Swedish twin registry	The American Medical Association Education and Research Foundation No 353	27,214	Feb. 1 1975-Jan.31 1976

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
		$	
Maintenance of the twin registries	National Swedish Environment Protection Board	18,300	July 1 1975-June 31 1976
Epidemiological studies on the new Swedish twin registry	The Council for Tobacco Research	43,923	July 1 1975-June 31 1977

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Lars Friberg

Signature [signature] Date 75 06 17

Telephone 08 (Area Code) 34 05 60 (Number) 1165 (Extension)

Checks payable to

The Karolinska Institute

Mailing address for checks

The Karolinska Institute

S-104 01 Stockholm, Sweden

Responsible officer of institution

Typed Name Margareta Almling

Title Head of Administration

Signature [signature] Date 75 06 17

Telephone 08 (Area Code) 34 05 60 (Number) 1123 (Extension)

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Enclosure 2

General outline of proposed research

The Twin Research Program at the Department of Environmental Hygiene of the Karolinska Institute as well as of the Swedish National Environment Protection Board was started in 1959 by the compilation of a twin registry covering all twin pairs of the same sex, born between 1886 and 1925 and still living in the country at the time of compilation (about 11 000 complete pairs). The main reason for setting up the registry was to find twin pairs with different environmental exposures, such as different smoking habits. The aim was to follow such twin pairs for a long period of time and to analyze some disease symptoms and signs as well as subsequent mortality in relation to e.g. different types and amounts of smoking. The research has been concentrated upon smoking but a large variety of other items has been studied at the same time, partly because of the confounding correlation of these items to the smoking habit, and partly because of interest in their own effects. The registry has also been used for some special clinical investigations, relating mostly to the effect of smoking and other behavioral factors in connection with ischemic heart disease.

The results from the registry have been published in medical journals and have also appeared as monographs (a list of publications is attached as enclosure 3). A comprehensive report is now being prepared, partly as a guide line for researchers who want to embark on twin studies and partly as a summary of the highlights of results hitherto obtained within the frame of the Swedish research. This report will appear in 1976 and is financially supported by the CTR.

Of main interest for the present application are the results from the twin registry in regard to mortality. An 11-year follow-up study was published in 1973 (enclosure 1) and updated reports (enclosure 1, 4, 5) have subse-

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quently been submitted to the American Medical Association Education and Research Foundation, which provided the funding. As these reports provide details in regard to methodology and data validity only a very brief summary of the main results will be given here.

The analysis was approached in two ways, first as a "conventional" epidemiological study on the twins without any regard paid to their pairing. The results showed the usual relationship between smoking and gross mortality, displaying relative risk of about 2-2.5 for male smokers of 10+ cigarettes a day; the corresponding figure for women was about 2.

The second approach was an analysis of the smoking discordant twin pairs. It was shown that among 706 male dizygotic smoking discordant pairs 55 deaths or "first deaths" occurred in a high smoking group in comparison to 31 such deaths in a low smoking group, i.e. a relative risk of 1.8. Among 246 corresponding monozygotic male pairs, the numbers were 18 versus 18, giving a ratio of unity. About the same dissimilarity between the dizygotic and monozygotic group was also seen in females. Unpublished data from the extended follow-up through June 1974 (enclosure 5) showed 71 deaths in a high smoking group and 46 deaths in a low smoking group among dizygotes, while the corresponding figures in the monozygotic group were 23 versus 22, still counted as deaths or "first deaths". The numbers become very small and show a considerable statistical variation if broken down by age and by varying degrees of smoking discordance. The hypermortality in the dizygotic groups was mainly due to coronary heart disease, lung cancer, suicides and accidents.

The 1973 publication also considered alcohol habits, reflected as a registration in a nationwide registry comprising people who had been in conflict with the society due to misdemeanors in connection with alcohol abuse. It was found that among the nonsmokers in the

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study 8-10 percent were so registered as against 25-30 percent among smokers of 10+ cigarettes a day. The mortality among the registered subjects showed up to be significantly higher than the nonregistered already in the nonsmoking group, i.e. 2.7 versus 4.3 percent among males born 1911-1925, and 10.2 versus 17.3 percent among males born 1901-1910. In 4 och 6 comparisons within smoking groups the mortality was higher among the registered. These findings directly point to the fact that the smoking effect cannot be evaluated unless drinking habits are controlled.

It has not yet been possible to examine to any great extent the confounding effect of social and habitual variables other than drinking in the rather limited series of discordant twins, even though the importance of such factors has been demonstrated on a non-pair basis in the total sample (enclosures 1, 4, 5).

It is considered of very great importance, however, that the mortality study be continued until such numbers of death are reached that will allow closer analysis. It shall be pointed out that the number of deaths can be expected to increase considerably during the coming 3 years, depending on the age structure of the twin series. Up to June 1974 2 959 deaths have occurred and another 1 990 deaths are expected to accur up to June 1978. Among those born 1901-1925, 1 393 deaths accurred up to June 1974 and another 1 026 deaths are expected to occur up to June 1978.

This project has been funded during 1971 to 1975 by a 5 year grant from the American Medical Association Education and Research Foundation, as well as by intramural funds from the Karolinska Institue and the Swedish National Environment Protection Board. As the AMA agency has now terminated its operation we are hereby requesting corresponding financial support from the Council for Tobacco Research - USA, Inc. for the time period 1976-1978.

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STUDIES ON THE SWEDISH TWIN REGISTRY Enclosure 3

Year	No	Author	Title and publication
1959	64	Friberg, L Kaij, L Dencker, S J Jonsson, E	Smoking Habits of Monozygotic and Dizygotic Twins. Brit. Med. J. 1959, I:1090-1092
1961	103	Cederlöf, R Friberg, L Jonsson, E Kaij, L	Studies on Similarity Diagnosis in Twins with the Aid of Mailed Questionnaires. Acta Genet. 11:338-362, 1961.
1963	134	Cederlöf, R Friberg, L Jonsson, E Kaij, L	Morbidity among Monozygotic Twins with Reference to Smoking Habits and Place of Residence. Preprint, Stockholm 1963.
1964	145	Cederlöf, R	Tvillingregistret. Preliminärt meddelande Nord. Hyg. Tidskr. 45:63-70, 1964.
	146	Cederlöf, R Friberg, L Jonsson, E Kaij, L	Sjuklighet hos enäggstvillingar med hänsyn till rökvanor och bostadsort. Nord. Hyg. Tidskr. 45:71-75, 1964.
1965	157	Cederlöf, R Friberg, L Jonsson, E Kaij, L	Morbidity among Monozygotic Twins. Arch. Environ. Health 10:346-350, 1965.
1966	170	Lundman, T	Smoking in Relation to Coronary Heart Disease and Lung Function in Twins. Acta Med. Scan. 180: Suppl. 455, 1966. Akad. avhandling.
	184	Cederlöf, R	The Twin Method in Epidemiological Studie on Chronic Disease. Stockholm 1966, Akad. avhandling.
1967	186	Cederlöf, R Friberg, L Jonsson, E Kaij, L	Respiratory Symptoms and "Angina Pectoris in Twins with reference to Smoking Habits An Epidemiological Study with Mailed Questionnaire. Arch. Environ. Health. 13:726-737, 1966
	187	Cederlöf, R Jonsson, E Lundman, T	On the Validity of Mailed Questionnaires in Diagnosing "Angina Pectoris" and and "Bronchitis". Arch. Environ. Health 13:738-742, 1966.
	188	Cederlöf, R	Urban Factor and Prevalence of Respirator Symptoms and "Angina Pectoris". A Study o 9,168 Twin-Pairs with the Aid of Mailed Questionnaires. Arch. Environ. Health 13:743-748, 1966.

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Year	No	Author	Title and publication
1967	190	Cederlöf, R Friberg, L Jonsson, E	Hereditary Factors and "Angina Pectoris". A Study on 5,877 Twin-Pairs with the Aid of Mailed Questionnaires. Arch. Environ. Health 14:397-400, 1967.
	191	Cederlöf, R Edfors, M-L Friberg, L Jonsson, E	Hereditary Factors, "Spontaneous Cough" and "Smoker's Cough". A Study on 7,800 Twin-Pairs with the Aid of Mailed Questionnaires. Arch. Environ. Health 14:401-406, 1967.
1968	215	Cederlöf, R Friberg, L	Tobaksrökning och hälsa. Resultat från epidemiologiska tvillingundersökningar. Läkartidningen 65:2727-2734, 1968.
	227	Jonsson, E Nilsson, T	Alkoholkonsumtion hos monozygota och dizygota tvillingpar. Nord. Hyg. Tidskr. 49:21-25, 1968.
1969	246	Cederlöf, R Friberg, L Hrubec, Z	Cardiovascular and Respiratory Symptoms in Relation to Tobacco Smoking. A Study on American Twins. Arch. Environ. Health 18:934-940, 1969.
	249	Liljefors, I Piscator, M Risinger, C	Exercise Proteinuria in Monozygotic and Dizygotic Twins. Medicine and Sport 3:333-339, 1969.
1970	266	Liljefors, I	Coronary Heart Disease in Male Twins. Hereditary and environmental factors in concordant and discordant pairs. Stockhol 1970. Akad. avhandling.
	277	Friberg, L Cederlöf, R Lundman, T Olsson, H	Mortality in Smoking Discordant Monozygotic and Dizygotic Twins. A Study on the Swedish Twin Registry. Arch. Environ. Health 21:508-513, 1970.
1971	281	Cederlöf, R Floderus, B Friberg, L	Cancer in MZ and DZ Twins. Acta Genet. Med. Gemellol. 19:69-74, 1970.
	282	Cederlöf, R Floderus, B Friberg, L	The Swedish Twin Registry. Past and futur use. Acta Genet. Med. Gemello. 19:351-354 1970.
	284	Cederlöf, R Epstein, F H Friberg, L Hrubec, Z Redford, E P	Twin Registries in the Study of Chronic Disease with Particular Reference to the Relation of Smoking to Cardiovascular and Pulmonary Diseases. Repl on and Internat. Symp. in San Juan, Puerto Rico, 1-4 Dec. 1969. Acta. Med. Scan. Suppl. 523, 1971.
	286	Lundman, T Liljefors, I Cederlöf, R Friberg, L	The Validity of the Questionnaire Diagnosis "Angina Pectoris". Arch. Environ. Health 22:597-599, 1971.

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Year	No	Author	Title and publication
1971	292	Edfors-Luba, M-L	Allergy in 7,000 Twin Pairs. Acta Aller. 26:249-285, 1971.
	294	Cederlöf, R Kaij, L	The Effect of Childbearing on Bodyweight A Twin Control Study. Acta Psychiat. Sca 46: Suppl. 219:47-49, 1970.
	295	Cederlöf, R	The Use of Twin Studies in CNSLD. Ur Bro chitis III. Proc. of the 3:rd internat. Symposium on Bronchitis at Gronningen, 23-26 Sept. 1969, pp 120-127.
1972	324	Camner, P Philipson, K Friberg, L	Tranchebronchial Clearance in Twins. Arc Environ. Health 24:82-87, 1972.
	333	Camner, P Philipson, K	Tracheobronchial Clearance in Smoking-Discordant Twins, Arch. Environ. Health 25:60-63, 1972.
	339	Helander, E	Om frekvensen av fylleristraff, särskilt hos tvillingar. Socialmedicinsk tidsskr. 49:555-561, 1972.
1973	369	Hrubec, Z Cederlöf, R Friberg, L Horton, R Ozolins, G	Respiratory Symptoms in Twins. Arch Environ Health 27:189-195, 1973.
	390	Friberg, L Cederlöf, R Lorich, U Lundman, T de Faire, U	Mortality in Twins in Relation to Smoking Habits and Alcohol Problems. Arch. Envirc Health 27:294-304, 1973.
1974		Myrhed, M	Alcohol Comsumption in Relation to Factor Associated with Ischemic Heart Disease. Acta Medica Scand. Suppl. 567, Akad. avh.
		Floderus, B	Psycho-social Factors in Relation to Coronary Heart Disease and Associated Risk Factors. Nord. Hyg. Tidskr. Suppl. 6 Akad. avhandling.
		de Faire, U	Ischemic Heart Disease in Death Discordan Twins. Acta Medica Scanc. Suppl. 568. Akad. avhandling.
1975		Medlund, P Cederlöf, R Floderus, B Friberg, L Sörensen, S	The New Swedish Twin Registry. A registry of births for 110,000 twins and of addres ses for 80,000 twins born 1926-27 plus basedata on environmental medicine factor for 32,000 twins (13,700 complete same-sexed pairs) born 1926-1958.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 9, 1975

Grant Application No. 1047

PHARMACOLOGY

TO: The committee comprising Drs. Bing, Jacobson, Sommers and Wyatt

SUBJECT: Mario D. Aceto, Ph.D., Medical College of Virginia, Richmond, Virginia
New application No. 1047
"Antinicotinic Effects and Antianxiety Agents"

History

An informal inquiry was handled as Case No. 313. The Executive Committee voted to encourage application.

Request

Application No. 1047 requests $39,808 for the first year of a two year project: the estimate for the second year is $26,908.

Documents submitted (attached)

Application dated June 30, 1975 (15 pages, including CVs of Drs. Aceto, Dewey and Harris).

David Stone

DS/lp
Encls.

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1047

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 7 - 1975
INSTITUTE

Application for Research Grant
(Use extra pages as needed)

Date 6/30/75

1. Principal Investigator (give title and degrees).

Mario D. Aceto, Ph.D., Associate Professor of Pharmacology

2. Institution & address:

Medical College of Virginia
Virginia Commonwealth University
Health Sciences Division
MCV, Box 726
Richmond, VA 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Antinicotinic Effects and Antianxiety Agents

5. Proposed starting date: Jan. 2, 1976

6. Estimated time to complete. 2 years

7. Brief description of specific research aims:

1) Determine the relative localization of nicotine-^{14}C over a wide dose range in selected rat brain areas.

2) Determine the subcellular distribution of nicotine-^{14}C in the rat brain.

3) Study the effects of antianxiety agents such as librium and meprobamate upon nicotine-^{14}C localization.

4) Attempt to ascertain a functional role for the central nicotinic nervous system as it relates to the mechanism of action of antianxiety agents.

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8. Brief statement of working hypothesis:

Although it has been shown that cholinergic receptors in the central nervous system (CNS) may be either nicotinic or muscarinic (Eccles, 1964), little work has been done on the localization of nicotine in the brain and on its subcellular distribution (Larson and Silvette, 1964, 1968, 1971). Even less is known about the role of the nicotinic nervous system and its interactions with CNS drugs. Studies by Hansson and Schmiterlow (1962) have shown that soon after the administration of nicotine-methyl-^{14}C in mice, very high concentrations of nicotine appeared in the CNS. Studies by the investigator (Aceto, 1967) showed that for the intraventricular (intracerebral) route of administration that a direct relationship between ganglion blocking potency and blocking of nicotine extensor convulsions existed and that the site of nicotine extensor convulsions is central in origin and is associated with brain areas near the ventricles. Later in 1971, Benesova and Nahunek reported a correlation between the degree of antinicotine convulsant activity and the clinical efficiency of antidepressants in agitated depression. These studies encouraged the author to examine the possible relationship of the antinicotine effects of a wide variety of CNS agents to their clinical properties. A good relationship was found between blockage of nicotine extensor convulsions and sedative antianxiety properties (Aceto, 1975, accepted for publication in Pharmacology). This relationship was especially good for drugs designated as antidepressants, antipsychotics and antianxiety agents. Because it was shown that for the drugs classified as antianxiety agents, there was a direct relationship between the recommended therapeutic dose in man and antinicotine potency in the mouse, this study will focus on this relationship.

9. Details of experimental design and procedures:

The brain areas which we propose to investigate are the cortex, and cerebellum. The subcellular fractionation procedures described below are currently being used in this laboratory, and are primarily based on those reported by DeRobertis et al., 1962; Mule et al., 1961; and Hokin and Hokin, 1958.

I. SUBCELLULAR FRACTIONATION OF BRAIN TISSUE (DeRobertis et al., 1962; Mule et al., 1967)

Brain tissue is homogenized with a teflon pestle for two minutes at a speed of 400 rpm. The homogenate is diluted with 0.32 M sucrose (Ca++) to give a final concentration of 1 g of brain per 10 ml. An aliquot representing 10% of the total homogenate is removed and labelled homogenate. The remaining homogenate is centrifuged at 900 times g for 10 minutes at 0°C in a Sorvall RC2-B centrifuge. This centrifugation yields a crude nuclear pellet. The supernatant is decanted and the crude nuclear pellet is washed twice. The resulting suspension is then centrifuged at 900 times g for 10 minutes at 0°C. The supernatant is decanted and combined with the other supernatant. The final crude nuclear pellet consists of nuclei, myelin, membrane fractions and tissue debris.

The combined crude nuclear supernatants are centrifuged in the Sorvall RC2-B centrifuge at 11,500 times g for 20 minutes at 0°C to yield a crude mitochondrial pellet which contains mitochondrial nerve endings, membrane fragments and myelin. The supernatant is decanted, and the pellet is washed once with 0.32 M sucrose (Ca++) and centrifuged at 11,500 times g. The pellet wash is added to the supernatant. The final crude mitochondrial pellet is resuspended in a vol-

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ume of 0.32 M sucrose equivalent to 1/3 of the original homogenate volume. The combined supernatants are centrifuged for 30 minutes in the Beckman L3-50 ultracentrifuge at 124,000 times g. The supernatant is decanted and referred to as the final soluble supernatant fraction. The pellet, which is the microsomal pellet, is resuspended in 0.32 M sucrose (Ca++) for marker assays or in 0.1 N HCl for radioactive counting.

II. SUBFRACTIONATION OF THE CRUDE MITOCHONDRIA

In order to isolate nerve endings and mitochondria, a discontinuous sucrose density gradient is prepared by layering 0.8 M, 1.0 M, 1.2 M and 1.4 M sucrose in 17-ml cellulose nitrate tubes. An aliquot of the crude mitochondrial fraction is layered on top. The tubes are placed in the Beckman L3-50 ultracentrifuge and spun at 81,000 times g for 120 minutes. The following layers are obtained: myelin, membrane fractions, cholinergic nerve endings, non-cholinergic nerve endings, and free mitochondria.

III. OSMOTIC SHOCK OF NERVE ENDINGS AND ISOLATION OF SYNAPTIC VESICLES

Synaptic vesicles are isolated from nerve endings by diluting an aliquot of the crude mitochondrial (CM) fraction with 10 uM $CaCl_2$ to make a final 0.32 M sucrose solution. The diluted CM is homogenized for 2 minutes at 400 rpm. The homogenate is centrifuged in the Sorvall RC2-B centrifuge at 11,500 times g for 20 minutes at 0°C. The pellet (M_1) consists of swollen mitochondria, myelin fragments, and the subsynaptic web. The supernatant is centrifuged at 124,000 times g for 30 minutes at 0°C. The pellet consists primarily of synaptic vesicles (M_2). The supernatant is considered to be the final soluble supernatant (M_3).

IV. SUBFRACTIONATION OF THE CRUDE NUCLEAR PELLET

In order to isolate pure nuclei, a discontinuous sucrose density gradient with 0.8 M and 1.2 M sucrose is prepared in a 17 ml cellulose nitrate tube by adding 6.0 ml of 1.2 M sucrose and carefully layering 6.0 ml of 0.8 M sucrose on top. 5.0 ml or less of the crude nuclear pellet which has been resuspended in 0.32 M sucrose is then layered on top and spun in the Beckman ultracentrifuge at 81,000 times g at 0°C for 120 minutes. The top layer, designated N_1, consists of large myelin fragments. The middle layer, N_2, consists primarily of nuclei, but contains some mitochondria, myelin fragments and synaptic vesicles. The pellet N_3 contains whole cells, tissue debris and blood cells.

V. ISOLATION OF DNA, RNA AND PHOSPHOLIPID FROM THE SUBCELLULAR FRACTION OF RAT BRAIN

Brain tissue for marker assays is homogenized and subfractionated as described above. Twenty ml of the supernatant and 5-10 ml of each of the remaining subcellular fractions are mixed with an equal volume of 10% TCA in a 45 ml polypropylene centrifuge tube. The samples are centrifuged in the Sorvall RC2-B at 12,000 times g for 5 minutes at 0°C. The liquid is discarded by decantation and the pellets washed four times with cold 5% TCA. Each washing consists of adding cold 5% TCA and resuspending the pellet by gentle stirring with a plastic rod. The suspension is centrifuged at 12,000 times g for 5 minutes and the liquid discarded. To the final pellets are added cold 5% TCA, and the pellets are resuspended by gentle stirring with a plastic stirring rod. One-half of this suspension is removed and placed in a 15 ml polyethylene centrifuge tube for DNA assay. The remaining suspension is centrifuged at 12,000 times g for 5 minutes. The supernatant is decanted and discarded. The pellet

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is then suspended in ethanol and $CHCl_3$ is added. The samples are stirred by Vortex until all material dissolves. The tubes are tightly stoppered and stored overnight at 4°C. One then adds 0.1 N HCl (cold) and shakes to form an emulsion. The emulsion is centrifuged in the Sorvall RC2-B at 30,000 times g for 5 minutes at 0°C. The aqueous layer contains the RNA, and the chloroform layer contains the phospholipids.

VI. CHEMICAL AND ENZYMATIC MARKER ASSAYS

In order to verify the morphology of each fraction, the following chemical and enzymatic assays are performed:

1. Protein is determined by the method of Lowry (1951).
2. DNA is estimated by the diphenylamine reaction described by Burton (1956).
3. RNA is estimated by Schneider's RNA assay (1957).
4. Phospholipid phosphorus is determined by the method described by Bartlett (1959).
5. Succinic dehydrogenase activity is determined by the method of Bonner (1955).
6. NADPH-cytochrome-C-reductase activity is determined as described by Mule (1967).

DNA, RNA and phospholipid are isolated for assay as described previously.

VII. DETECTION OF RADIOACTIVITY AND IDENTIFICATION OF NICOTINE

An aliquot of the crude mitochondrial fraction is subjected to osmotic shock, rehomogenization and centrifugation in order to isolate synaptic vesicles. An aliquot of each fraction that contains radioactivity is oxidized in a Packard Tri-Carb Sample Oxidizer. The samples are counted in a Beckman scintillation counter and corrected for quenching by external standardization. To determine how much of the radioactivity is due to unchanged drug or metabolites, thin-layer and gas chromatography of organic extracts is done.

VIII. ANIMAL STUDIES

The research plan is to first determine the disposition of nicotine-^{14}C in male rats. Nicotine-^{14}C will be given intravenously to six naive animals per dose and at least three doses of nicotine will be studied. At 5 and 20 minutes after nicotine-^{14}C, the animals will be sacrificed and the brain levels and subcellular disposition of nicotine will be determined. In the drug studies, animals will also be injected subcutaneously with selected antianxiety agents such as diazepam, chlordiazipoxide and meprobamate (each drug will be given at three dose levels and at two different time periods; namely, ½ and 2 hours) before receiving radioactive nicotine. Saline controls will be run with each drug group and the mean concentration of nicotine-^{14}C in the various brain areas and subcellular fractions will be determined and expressed as pmol/g $\pm$ S.D. or as % of control and an analysis of variance of the data will be done with each drug. These results will be used to interpret the possible involvement and function of the nicotine nervous system as it relates to antianxiety agents. For the localization studies, 50 to 100 mg portions of selected brain areas will be oxidized in the Packard Oxidizer and the radioactivity counted in the Beckmann scintillation instrument. In the subcellular experiments, the selected brain areas will be pooled to yield sufficient tissue for the studies (2 g.).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Department of Pharmacology is currently occupying 20,000 square feet of space. The department has a library which makes the important journals available for the members. In addition, a new Health Sciences Library has been built in close proximity to the Department of Pharmacology.

The Department of Pharmacology has its own animal facilities and they have recently been refurnished. Attendents for the care of the animals are supported by the department.

The CNS Division of the Department of Pharmacology occupies a space of 8,000 square feet with office and research space available. These laboratories are well supplied with pharmacological equipment. The proposed study will be conducted in one of these laboratories.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

Biographical Sketch

Name: Aceto, Mario D. Role in Project: Principal Investigator

Date of Birth: REDACTED Marital Status: REDACTED

Education: University of Rhode Island, Kingston, R.I. - B.S. Pharmacy -
University of Maryland, College Park, MD. - M.S. Pharmacology - 1955
University of Connecticut, Storrs, Conn. - Ph.D. Pharmacology - 1959

Professional Experience:

1973 -	Associate Professor of Pharmacology, Medical College of VA.
1973 - 1973	Project Head, Sterling-Winthrop Research Institute
1967 - 1973	CNS Section Head and Project Leader, Sterling-Winthrop Research Institute
1966 - 1973	Senior Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1964 - 1966	Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1963 - 1966	Group Leader (Pharmacology) Sterling-Winthrop Research Institute
1964 - 1972	Lecturer in Pharmacology, Albany Medical College
1962 - 1963	Associate Research Biologist (Pharmacology) Sterling-Winthrop Research Institute
1959 - 1962	Assistant Professor, University of Pittsburgh
1958 - 1959	Instructor, Pharmacology, University of Pittsburgh
1956 - 1958	Graduate Assistant, University of Connecticut
1953 - 1956	Graduate Assistant, University of Maryland

Honors: Honor Achievement Award granted by the American College of Angiology for the top animal study published during a 5 year period in Angiology in June, 1966. REDACTED

Publications:

Aceto, M.D., Harris, L.S., Dewey, W.L. and Balster, R.L.: Dependence studies of new compounds in rhesus monkeys. Committee on Problems of Drug Dependence, 1975. (In Press).

Aceto, M.D. and Harris, L.S.: Comparative study of the effects of two narcotic antagonists naloxone and nalorphine on developing dependence in rhesus monkeys. Accepted for publication in J. Pharmac. Exptl. Ther. (1975).

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Aceto, M.D., Harris, L.S., Balster, R.L., and Dewey, W.L.: Evaluation of dependence liability of narcotic agonist and antagonist. Committee on Problems of Drug Dependence 77-78, 1974.

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Pearl, J., Aceto, M.D.G., and Fitzgerald, J.J.: Drugs and avoidance performance Psychon. Sci., 6: 41-42, 1966.

Harris, L.S., Pearl, J., and Aceto, M.D.G.: Similarities in effects of barbiturates and mild tranquilizers on activity in mice. Psychon. Sci., 4: 267-268, 1966.

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R: REDACTED MATERIAL

Biographical Sketch

Name: Dewey, William L. Role in Project: Co-Investigator

Date of Birth: REDACTED Martial Status: REDACTED

Education:

St. Bernardine of Siena College, Loudonville, N.Y.	- B.S.	Biology - 1957
College of Saint Rose, Albany, N.Y.	- M.S.	Biology - 1964
Univ. of Connecticut, Storrs, Conn.	-Ph.D.	Pharmacology - 1967

Professional Experience:

1973 -	Associate Professor Dept. of Pharmacology, Medical College of Va., Richmond, Va.
1969 - 1973	Assistant Professor of Pharmacology, University of North Carolina, Chapel Hill, N.C. 27514
1971 - 1973	Consultant, Sharps Associates, 767B Concord Ave., Cambridge, Mass.
1968 - 1969	Instructor of Pharmacology, University of North Carolina, Chapel Hill, N.C. 27514
1967 - 1970	Consultant, Arthur D. Little Inc., Cambridge, Mass.
1967 - 1968	Postdoctoral Research Trainee. Neurobiology Program (MH-1107-01) University of North Carolina, Chapel Hill, N.C.
1966 - 1967	Postdoctoral Research Fellow, Dept. of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, N.C. 27514
1964 - 1966	Graduate Assistant, School of Pharmacy, University of Connecticut
1959 - 1964	Assistant Research Biologist, Sterling-Winthrop Research Institute Rensselaer, N.Y.

Honors: REDACTED

Publications:

Aceto, M.D., Harris, L.S., Dewey, W.L., and Balster, R.L.: Dependence studies of new compounds in the rhesus monkey (Submitted for publication.)

Dewey, William L., Patrick, Graham A. and Harris, Louis S.: Annual Report: Narcotic Antagonists in the rat infusion technique (submitted for publication.)

Dewey, William L. (Book Review) Narcotics and the hypothalamus, Kroc Foundation Symprsia No 2. Zimmerman and Gerge editors. Amer. J. Pharm. Ed. (in press).

Spaulding, T.C. and Dewey, W.L.: Some effects of the behaviorally active drug, phenitrone a purported hashish and LSH antagonist, on brain noradrenergic and serotonergic systems. Res. Comm. Chem. Path. and Pharm. (in press).

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Chipkin, R.E., Dewey, W.L., Harris, L.S. and Lowenthal, W.: Effect of propranolol on antinociceptive and withdrawal characteristics of morphine. Pharmacology, Biochemistry, and Behavior (in press).

Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.S.: Subcellular and tissue distribution of H3-delta-9-tetrahydrocannabinol in brain and peripheral organs of nontolerant and tolerant dogs (Submitted for publication).

Harris, L.S. and Dewey, W.L.: Narcotic and other strong analgesics, narcotic antagonists, and antitussives, in ESSENTIALS OF PHARMACOLOGY, J.A. Bevan (ed.), Harper & Row, New York, 1975 (in press).

Martin, B.R., Dewey, W.L., Harris, L.S. and Beckner, J.: Marihuana-like activity of new synthetic tetrahydrocannabinols. Pharmacology, Biochemistry and behavior (in press).

Martin, B.R., Dewey, W.L., Harris, L.S. and Beckner, J.S.: H3-Delta-9-tetrahydrocannabinol distribution in pregnant dogs and their fetuses (Submitted for publication).

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R: REDACTED MATERIAL

Biographical Sketch

Name: Harris, Louis S. Role in Project: Co-Investigator

Date of Birth: REDACTED Marital Status: REDACTED

Education:

Harvard College, Cambridge, Mass.	- B.S.	Chemistry - 1954
Harvard University, Cambridge, Mass.	- M.S.	Medicinal Sci. - 1956
Harvard University, Cambridge, Mass.	- Ph.D.	Pharmacology - 1958

Professional Experience:

1972 -	Professor and Chairman, Pharmacology, Medical College of Va.
1970 -	Professor of Pharmacology, University of North Carolina School of Medicine, and School of Pharmacy
1969 -	National Institute of Mental Health Psychotomimetic Agents Committee-Chairman, 1971 -
1969 -	Editorial Board, Journal of Pharmacology and Experimental Therapeutics
1966 - 1970	Associate Professor of Pharmacology, University of North Carolina School of Medicine and School of Pharmacy
1962 - 1966	Section Head in Pharmacology and Senior Research Biologist, Sterling-Winthrop Research Institute
1961 - 1962	Associate Member, Sterling-Winthrop Research Institute
1960 - 1961	Research Biologist, Sterling-Winthrop Research Institute
1959 - 1966	Lecturer in Pharmacology, Albany Medical College
1958 - 1960	Research Associate, Sterling-Winthrop Research Institute
1955 - 1958	National Institutes of Health, Fellow
1954 - 1955	National Science Foundation, Fellow
1951 - 1952	Research Assistant in Anesthesiology, Massachusetts General Hospital

Honors: Winner of Martius Yellow Competition - Department of Chemistry, Harvard College, 1958, REDACTED

Publications:

Dewey, W.L., Patrick, G.A., and Harris, L.S.: Annual Report: Narcotic' antagonist in the rat infusion technique. Committee on Problems of Drug Dependence, 1975.

Aceto, M.D., Harris, L.S., Dewey, W.L., and Balster, R.L.: Dependence studies of new compounds in the rhesus monkey. Committee on Problems of Drug Dependence, 1975.

Chiplin, R.E., Dewey, W.L., Harris, L.S., and Lowenthal, W.: Effect of propranolol on antinociceptive and withdrawal characteristics of morphine. Submitted to Pharmacology, Biochemistry and Behavior.

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Publications (continued)

Dewey, W.L., Martin, B.R., and Harris, L.S.: Chronic effects of Δ^9-THC in animals: Tolerance and biochemical changes. Submitted

Patrick, G.A., Dewey, W.L., Spaulding, T.C., and Harris, L.S.: Relationship of brain morphine levels to analgesic activity in acutely treated mice and rats and in pellet-implanted mice. Submitted to JPET.

Dewey, W.L. and Harris, L.S.: The tail flick test. In: Methods in Narcotics Research, S. Eherpreis (ed.), Marcel Dekker, Inc. (In press).

Adams, M.D., Earnhardt, J.T., Dewey, W.L., and Harris, L.S.: Vasoconstrictor actions of Δ^8- and Δ^9-tetrahydrocannabinol in the rat. Submitted to JPET.

Pars, H.G., Granchelli, F.E., Razdan, R.K., Rosenberg, F., Teiger, D. and Harris, L.S.: Nitrogen analogs of the Cannabinoids - chemistry and general pharmacology. Submitted to J. Med. Chem., 1975.

Harris, L.S., Munson, A.E., and Carchman, R.A.: Anti-tumor properties of cannabinoids. International Conference on the Pharmacology of Cannabis, Savannah, Georgia, December 3-6, 1974.

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Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.: Marihuana-like activity of new synthetic tetrahydrocannabinols. In Press to Pharmacology, Biochemistry, and Behavior.

Munson, A.E., Levy, J.A., Harris, L.S., and Dewey, W.L.: Effects of Δ^9-tetrahydrocannabinol on the immune system. International Conference on the Pharmacology of Cannabis, Savannah, Georgia, December 3-6, 1974.

Munson, A.E., Harris, L.S., Friedman, M.A., Dewey, W.L., and Carchman, R.A.: Anti-neoplastic activity of cannabinoids. Submitted to J. National Cancer Institute.

Pedigo, N.W., Dewey, W.L., and Harris, L.S.: Determination and characterization of the antionociceptive activity of intraventricularly administered acetylcholine in mice. In Press to JPET.

Martin, B.R., Dewey, W.L., Harris, L.S., and Beckner, J.S.: Subcellular localization H^3-Δ^9-tetrahydrocannabinol in brain of nontolerance and tolerant dogs (Submitted to JPET).

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14. First year budget.

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
Aceto, Mario D.	Principal Investigator	25	
Dewey, William L.	Co-Investigator	5	
Harris, Louis S.	Co-Investigator	.1	
Technical			
To be recruited, Lab Specialist + 10.06 fringe benefits		100	REDACTED

Sub Total for A

B. Consumable supplies (by major categories)

Animals and animal care; chemicals; radiochemicals; glassware, etc.

Sub-Total for B 8,000.00

C. Other expenses (itemize)

Travel (Fed. Am. Soc. Expt. Biol.) - 350.00

Misc., Laundry, reprint charges, office supplies, maintenance of equipment -- 1000.00

Sub-Total for C 1,350.00

Running Total of A + B + C — REDACTED

D. Permanent equipment (itemize)

Beckman Liquid Scintillation LS 330 System (Dept. has an instrument, but demands on its use are heavy)

Sub Total for D 14,500.00

E. Indirect costs (15% of A+B+C) 3,301.00

Total request 39,808.00

15. Estimated future requirements:

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	REDACTED	8,000	1,350	--	3,509	REDACTED
Year 3	--	--	--	--	--	--

*Includes 1,399 fringe benefits.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Nicotine Nervous System Interaction with CNS Drugs	A.D. Williams Research Award (seed money)	1,600	11/75 - 11/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Mechanism Studies on Experimental Brain Edema		232,107	9/1/75 - 8/31/80

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Mario D Aceto

Signature Mario D Aceto Date 6/27/75

Telephone 804 (Area Code) 770-3861 (Number) (Extension)

Checks payable to

Mr. Walter P. Lossing
Comptroller-Treasurer

Mailing address for checks

Virginia Commonwealth University
Medical College of Virginia
1200 East Broad Street
Richmond, Virginia 23298

Responsible officer of institution

Typed Name John J. Salley, D.D.S., Ph.D.

Title Associate Vice President
Research and Graduate Affairs

Signature John J. Salley Date 7/2/75

Telephone 804 (Area Code) 770-7985 (Number) (Extension)

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#836B - ESSMAN

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 11, 1975

Grant application No. 836B

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner and Jacobson

Subject: Walter B. Essman, Ph.D., M.D., Queens College, Flushing, N.Y.
Continuation application No. 836B
"Metabolic Response to Stress - Tobacco Smoke Interactions"

History

This productive applicant has been supported by CTR through grants 623, 623A, and 623B (1968-1972) for "Study of Nicotine Action in Memory Consolidation". Grant No. 836 was awarded in September 1971 for a period of one year to undertake studies on the "Metabolic Response to Stress - Tobacco Smoke Interactions".

A three year plan (Grant No. 836A) was approved by the SAB in October 1972: Grant No. 836AR2 (second renewal) in the amount of $61,254 terminates on December 31, 1975.

The present application is to support a continuation of the previous studies.

Request

Application No. 836B requests $71,274 for the first year of a three year project. Estimates for the second and third years are $79,576 and $66,703, respectively.

Documents submitted (attached)

1. Application dated July 1, 1975 (17 pages).
2. C.V.s of Essman and Kornreich (3 pages)
3. Publications (relevant and recent)(2 pages).
4. Progress Report No. 3 for period July 16, 1974 - June 30, 1975 (7 pages).
5. Nine reprints.

D.S.

DS:wg
Atts.

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#836B

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

RECEIVED
JUL 1 1975

Application for Research Grant
(Use extra pages as needed)

Date: July 1, 1975

1. Principal Investigator (give title and degrees): Walter B. Essman, Ph.D., M.D.
Professor of Psychology & Biochemistry

2. Institution & address: Queens College of the City University of New York
65-30 Kissena Boulevard
Flushing, New York 11367
and
The Research Foundation of the City University of New York
1411 Broadway, New York, New York 10018

3. Department(s) where research will be done or collaboration provided:

Department of Psychology, Queens College of the City University of New York

4. Short title of study: "Metabolic Response to Stress--Tobacco Smoke Interactions"

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years.

7. Brief description of specific research aims: Several methods developed in our laboratory have enabled us to utilize cellular models in which specific biologically active molecules can be tested. Such molecules include both transmitter (norepinephrine, 5-Hydroxytryptamine, acetylcholine), or trophic (5-hydroxytryptamine, dopamine) agents, unique to such tissue. Specifically, developmental (age-dependent) and pathological (tissue changes) concommittant with acute and chronic stress stimuli have been studied. Their interaction with tobacco smoke and/or nicotine have been further considered. It is the purpose of this investigation to study tobacco smoke (including its gas phase) and biologically active constituents (nicotine) insofar as they interact with stress-induced stimuli in rodents.

Experimental description of the metabolic and molecular events associated with stress and its adaptation are investigated in several functional systems (brain, heart, lung, and gastro-intestinal tract).

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7. Brief description of specific research aims: (Continued)

The biologically active molecules altered by acute or chronic stress will be studied in the above mentioned tissue systems. Alterations in content, metabolism, uptake and synthesis will be studied with in vivo and in vitro methods in order to assess: 1) effects of acute or chronic cigarette smoke exposure and 2) acute and chronic effects of nicotine. The overall aim of this project is to provide a general description of those factors implicit in stress which cause molecular changes and how such effects are modified by interaction with nicotine.

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8. Brief statement of working hypothesis:

Previous research has shown that models of acute and chronic stress may be found in basic cellular mechanisms. The effects of tobacco smoke and/or nicotine can be measured in such model systems. On this basis, several working hypotheses have been formulated. The interaction of the effects of stress and nicotine alter the cellular and/or molecular effects produced by stress. The cellular mechanisms altered by stress will, upon appropriate presentation, show adaptation. On the basis of this general overall hypothesis, several specific working hypotheses have been developed: a) cellular changes in the central nervous system (storage, synthesis, and uptake of neurotransmitters by neurons, glia, and synaptosomes) are highly sensitive to and will occur with stress and nicotine; (b) pulmonary epithelial cells and pulmonary alveolar macrophages are sensitive to the effects of tobacco smoke. Differences in amine concentration in this tissue (epithelial cells) or amine interaction with tissue products (macrophages) will occur as a function of 1) age; 2) strain; 3) sex; and 4) the frequency and duration of tobacco smoke exposure; c) cardiac catecholamine uptake and release is altered by acute and chronic stress. Such alteration is dependent upon genetic differences and may be altered by cigarette smoke and/or nicotine. Experimentally induced diabetes mellitus or the use of catecholamine receptor antagonists will induce pathological changes in cardiac tissue. These changes will alter the effect of cigarette smoke upon amine metabolism of this tissue and will also change the pharmacological effect and tissue distribution of cardiolytic drugs; specifically, digoxin; (d) gastrointestinal tissue and its 5-HT containing storage depots will show morphological as well as biochemical changes in the development of stress-induced gastric ulcers. Such stress-induced changes and their recovery are sensitive to the effects of cigarette smoke. The stress-induced pathological changes may be predicted by changes in 5-HT; this relationship will serve as a basis for other predictions of effect from cigarette smoke and/or nicotine.

The working hypotheses underlying this proposal, therefore, concern themselves with tobacco smoke (nicotine) - stress-induced interactions as they apply to four tissue levels, the cellular and organelle specificity of which will reflect differences in response to systemic stress and physiological levels of nicotine.

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9. Details of experimental design and procedures

A large number of stressor agents and/or events have been utilized to produce physiological changes (Selye, 1950). There are several stressor conditions which have been reliably used in rodents (rats, mice, and guinea pigs); for acute stress, both restraint and electroshock will be utilized. Both of these stressor conditions can be varied as to (a) duration, (b) frequency, (c) time of day at which they are initiated and (d) age of the animal as well as its strain. Chronic stress will be provided through isolation housing; this stress and its sequalae has been extensively documented in experiments carried out in this laboratory for more than 10 years. Isolation stress can be titrated in terms of duration and can be carried out for animals of different ages as well as strain.

The proposed experiments will deal with peripheral (pulmonary, cardiac, and gastrointestinal) as well as central nervous system cellular and subcellular changes which accompany or follow either acute or chronic stress; the extent to which these effects interact with tobacco smoke and/or nicotine will be described. In peripheral tissues, the lung, heart and gastrointestinal tissue will be studied, specifically with regard to their relevant transmitter or trophic agents; i.e., 5-hydroxytryptamine, norepinephrine, or dopamine. The areas of the brain will be divided into functionally distinct regions; i.e., limbic region, basal ganglia, etc.

The proposed experiments will utilize both in vivo as well as in vitro techniques. The parameters to be specifically investigated include (1) the duration of stress; (2) the frequency with which stress stimulation is presented; (3) diurnal variations in the presentation of stress stimulation; (4) age differences in the effect of stress and (5) strain differences in the effects of stress. Exposure to tobacco smoke by means of a standardized nicotine content cigarette will be provided to an animal in a Walton Upright Cigarette Smoking machine; control animals will be exposed to filtered cigarette smoke (filtration

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of nicotine and exposure to gas phase only), or an equivalent volume and duration of room air provided to the smoking chamber. The parameters of the smoke exposure to be considered will include (a) frequency of smoke exposure, (b) duration of smoke exposure, and (c) time of day at which smoke exposure is provided.

Other parallel in vivo and in vitro studies will be carried out utilizing nicotine and several of its biologically active metabolites; these will be administered either in vivo along with the stress conditions, or will be utilized in vitro in those studies where tissue from stressed animals will be utilized. Nicotine will be given either as the alkaloid or in the bitartrate form. Nicotine metabolites will include those which in previous experiments have been clearly demonstrated to possess biological activity (Essman, 1973 (5)); (capable of being taken up by the cerebral cortex and basal ganglia). These compounds are cotinine, 3-pyridylacetic acid, and the analog, nornicotine. In vivo doses of these derivatives have been established in previous studies, and the in vitro concentration to be utilized will all be at 10^{-6}M, or less.

The following series of experiments will be specifically carried out for the tissue systems outlined.

I. Effects of Stress-Nicotine Interactions Upon Brain Tissue:

Studies with brain tissue will include a regional comparison of three major functional constituents -- neuronal cell bodies, glia, and synaptosomes. The regions of the brain to be considered will include (a) cerebral cortex, (b) diencephalon, (c) basal ganglia, (d) limbic region and (e) cerebellum. For each of these regions, standardized fractionation methods (differential and sucrose or sucrose-ficoll density gradient centrifugation) will be applied

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(Essman, 1973 (3)) or combined with newly developed methods; the latter techniques (i.e., isolation of neuronal perikarya from adjacent glia have been developed in our laboratory. These methods for cell separation yield highly enriched fractions of either neurons, glia or synaptosomes (synaptic nerve endings). The purity and morphology of each such fraction can be confirmed by the use of enzyme markers (acetylcholinesterase-neurons; butyrylcholineserase-glia) and/or scanning electron microscopy. The purity of these fractions will be verified with such markers and ulstrastructural correlates.

Thus, for brain tissue, it will be possible to assess the region in which the effects of stress and/or tobacco smoke are manifest, the cellular specificity of this effects, and the synaptic change produced. The basic experiments that will concern the regional, cellular and subcellular effects of stress-tobacco smoke interactions will involve:

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A) Specific Uptake of Biogenic Amines: This procedure will be carried out in vitro with the cell fractions (either neurons, glia, or synaptosomes) incubated in 1.25 µc per ml. of either C^{14}-labelled 5-hydroxytryptamine, norepinephrine, or dopamine for 45 minutes at 37°C in a standardized Krebs-Henselite medium ventillated with 95% O_2 and 5% CO_2. Measurement of the uptake of these biogenic amines will be made for the in vivo conditions of stress-tobacco smoke and/or nicotine interactions mentioned above. A further series of experiments will test nicotine or active metabolites thereof in vitro to assess effects upon amine uptake. Substantial data already obtained have indicated that concentrations of nicotine as little as 2.5×10^{-7} will exert highly significant effects on uptake. For example, 5-HT and norepinephrine uptake were reduced by nicotine in neurons and glia of the cerebral cortex. Diencephalic neurons had significantly increased catecholamine uptake stimulated by nicotine, but glia from that region

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showed significantly reduced catecholamine uptake.

Decreased cellular uptake of amines is usually an indication of enhanced excitability and is one of the mechanisms by which antidepressant drugs exert their effect. In this context, the excitatory effect of nicotine, in vitro, is consistent with the electrophysiological localization in vivo. The major differences between these similar effects, however, is that the concentrations required for electrophysiological changes are 100 to 1000 times greater. Therefore, the proposed methodology, aside from utilizing physiological concentrations of nicotine has also been shown sensitive to these low levels.

B) Cerebral Protein Synthesis: The in vitro incorporation of C^{14}-labelled leucine into proteins isolated from either neurons, glia, or synaptosomes isolated from several regions of the rodent brain has been reported (Essman, 1973 (6); 1974 (11)). After incubation (45 minutes under physiological conditions), proteins will be precipitated and assessed for incorporation of the labelled amino acid. These studies will evaluate stress-tobacco smoke interactions and assess the in vitro effects of nicotine and its metabolites. In the latter regard, nicotine concentrations as little as 2.5×10^{-7}M have been shown to alter amino acid incorporation into specific neuronal as well as glial proteins; for example, of several brain regions sampled, only the cells of the basal ganglia showed statistically significant increases in protein synthesis from nicotine (59% for neurons, 24% for glia). The increase in protein synthesis at the cellular level usually suggests excitation. The specificity of this effect from physiological concentrations of nicotine (basal ganglia) indicates the selectivity of the method for assessing the effects of nicotine at low levels.

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II. Effects of Stress-Nicotine Interactions Upon Pulmonary Tissue and Products:

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These experiments will consist of two basic protocols; the first will concern the effects of stress-tobacco smoke interactions upon the concentration and release of 5-hydroxytryptamine in pulmonary epithelial tissue; the second will concern in vivo stress and/or smoke effects upon the in vitro interaction of pulmonary alveolar macrophages with 5-hydroxytryptamine. These effects will be defined for age, sex, strain, and diurnal variations; all of these variables appear to contribute significantly to the effects of tobacco smoke and/or nicotine upon stress (Essman, 1974 (9, 10), 1975 (15). The tissue preparation, extraction and assay of 5-hydroxytryptamine has been previously described in numerous publications as well as progress reports from this laboratory; this involves standardized procedures for the spectrofluorometric determination of this biogenic amine (see Essman, recent publications).

The harvesting of pulmonary alveolar macrophages by bronchopulmonary lavage utilizes sterile Tullis solution maintained at a pH of 7.4; use of subsequent magnesium-free glucose medium precludes cellular replication. The number of macrophages utilized in the binding studies and their morphological characteristics will be assessed by scanning electron microscopy of fixed (gluteraldehyde) preparations. Binding studies with this preparation will utilize C^{14} labelled 5-hydroxytryptamine (0.1μc) in an equilibrium dialysis chamber through a 0.62 membrane for 16.5 hours. 5-HT binding per macrophage can be precisely calculated and related to the in vivo conditions (nicotine, gas phase, etc.) which produce qualitative changes in the macrophage. Such changes, already documented in preliminary studies include strain-related changes in the number of macrophage binding sites (increased in DBA mice, decreased in C3H mice) and macrophage elongation with increased furrowing. The in vitro effects of physiological concentrations of nicotine (10^{-7}M) and its metabolites upon 5-hydroxytryptamine binding by macrophage will be assessed by the addition of these compounds to the chamber

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before equilibrium dialysis is begun. Such factors as competition for the macrophage binding sites with nicotine will be determined using radiolabeled nicotine. No such studies have appeared in the experimental literature; their significance lies in the quantitative assessment of macrophage response (binding and morphology) following smoke exposure and induction of increased 5-HT by smoke. From preliminary data, it appears that increased or decreased macrophage binding sites for 5-HT only occur in those strains where pulmonary 5-HT is elevated by smoke. We believe that this relationship, clarified through the proposed experiments, will provide a novel definition of macrophage viability.

III. Effects of Stress-Nicotine Interactions Upon Cardiac Tissue:

Two general experimental procedures will be carried out with cardiac tissue. These will consist of both in vivo and in vitro experiments which relate stress and the effects of tobacco smoke, described above, to cardiac catecholamine uptake and to digoxin distribution. Previous studies have provided convincing evidence to show that cardiac norepinephrine (NE) uptake is severely reduced by stress (immobilization of mice for 8 minutes reduced cardiac NE uptake by 66%); reduced NE uptake may be reversed as adaptation to such stress (as measured by a successive decrease to normal of plasma corticosterone level) occurs; (e.g., after 21 days of successive episodes of immobilization, cardiac NE uptake had returned to within baseline limits). Animals, while immobilized, were exposed to 8 minutes of cigarette smoke for one week and showed only a 20% reduction in cardiac NE uptake. This finding suggests that cigarette smoke may exert a protective effect against stress-induced cardiac NE uptake reduction. The experiments will investigate (1) the in vivo uptake of norepinephrine (NE) and dopamine (DA) by cardiac tissue; (2) the in vitro uptake of these catecholamines by whole tissue and (3) uptake and distribution in subcellular fractions (nuclei, mitochondria, microsomes)

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thereof. Stress, acute (electroshock) as well as chronic (isolation), can markedly increase the cardiac distribution of digoxin (acute stress = 51%; chronic = 103%), an important cardiac glycoside (Essman, 1973 (8)). Both in vivo as well as in vitro studies will be conducted to assess changes in cardiac digoxin distribution resulting from stress-tobacco smoke interactions.

The significance of these studies lies in the potential functional impairment of cardiac activity by stress and its reversal by tobacco smoke Furthermore, digoxin toxicity appears to be potentiated by stress; tobacco smoke and/or nicotine may modify this effect.

Further studies will be carried out utilizing a model of experimental diabetes mellitus (EDM), induced in the rat by means of alloxan treatment (65 mg/kg i.v.). Such an experimental model has been tested by preliminary studies in this laboratory; fasting blood glucose levels are elevated by 2 weeks post-treatment, to approximately 280 mg%. The object of this animal model is to provide baseline data for a systemic stress condition wherein both cardiac catecholamine uptake and digoxin distribution may be assessed. Diabetic rats and normoglycemic controls will be exposed to tobacco smoke or treated with nicotine. The cardiac tissue will be fractionated into its subcellular constituents. In vitro studies will involve the effects of physiological levels of nicotine (10^{-7} - 10^{-5}M) and its active metabolites. Catecholamine uptake and digoxin distribution in the cardiac tissue and its organelles will be documented.

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Preliminary data from our laboratory have been obtained using normoglycemic (mean blood glucose = 105 mg%) and alloxan-induced hyperglycemic (mean blood glucose = 315 mg%) rats. Digoxin uptake was measured in vitro; the rate of uptake was significantly lower among the diabetic rats, and nicotine (10^{-5}M) lowered digoxin

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uptake of the cardiac tissue even more (Table 1).

Table 1

Uptake of H^3-Digoxin (cpm/mg protein)
by Cardiac Tissue from Normal and Diabetic Rats
as a Function of In Vitro Nicotine (10^{-5}M)

IN VIVO CONDITION	IN VITRO TREATMENT	
	0.9% NaCl	Nicotine
NORMOGLYCEMIC	124.4	114.2
HYPERGLYCEMIC	102.2*	91.8*

*p<.02

Norepinephrine uptake by cardiac tissue from diabetic rats was significantly reduced; nicotine (10^{-5}M) reduced cardiac norepinephrine uptake in normoglycemic rats, but did not alter the already reduced rate ofuptake in diabetic rats (Table 2).

Table 2

Uptake of C^{14}-Norepinephrine
(cpm/mg Protein) by Cardiac Tissue from Normal and
Diabetic Rats as a Function of In Vitro Nicotine (10^{-5}M)

IN VIVO CONDITION	IN VITRO TREATMENT	
	0.9% NaCl	Nicotine
NORMOGLYCEMIC	4988.2	1406.2*
HYPERGLYCEMIC	1286.5	1330.6

p<.01

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Cardiac mitochondrial protein synthesis was lowered in diabetic rats; nicotine lowered cardiac mitochondrial protein synthesis in normoglycemic rats, but in diabetic rats it led to increased synthesis (Table 3).

Table 3

Incorporation of C^{14}-Leucine Into
Proteins from Several Subcellular Fractions of
Rat Cardiac Tissue from Normal or Diabetic Animals: Effects of Nicotine

IN VIVO CONDITION	SUBCELLULAR FRACTION	IN VITRO TREATMENT	
		0.9% NaCl	Nicotine
NORMAL	NUCLEI	86.9	87.2
	MITOCHONDRIA	261.9	162.5*
	MICROSOMES	63.1	56.1
DIABETIC	NUCLEI	83.7	96.9
	MITOCHONDRIA	154.5	196.6*
	MICROSOMES	69.3	76.9

p<.02

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IV. Effects of Stress-Nicotine Interactions Upon Gastrointestinal Tissue:

The pathological change in gastrointestinal tissue with which the proposed experiments are concerned is the development of gastric ulcers in stressed animals. There have been no clear indications as to the significance of tobacco smoke upon ulcerogenesis. Several of our previous studies have indicated an etiological relationship between stress-induced gastric ulceration and gastrointestinal 5-hydroxytryptamine content and metabolism (Essman, et al., 1971); e.g. immobilization stress facilitated gastric ulcerogenesis in the pyloric region;

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stress-ulcerated mice had elevated gastric tissue pH and increased gastric 5-HT content.

This series of studies will measure 5-hydroxytryptamine content (utilizing standardized spectrofluorometric methodology) and turnover (utilizing steady-state kinetics methods) to evaluate the in vivo contributions of cigarette smoke exposure and/or nicotine treatment upon the development of gastric ulceration in mice. Histological changes (vasodilation, hemmorrhage, erosions, and infiltrations) in the gastric tissue will be evaluated. Experiments will be carried out to measure the biochemical (5-HT) as well as morphological changes in the gastrointestinal tract following (1) cessation of stress stimulation with continued tobacco smoke exposure or (2) in the absence of tobacco smoke exposure. The purpose of these experiments therefore, is to determine the extent to which tobacco smoke exposure or nicotine will contribute to a reversal of stress-induced gastric pathology after cessation of stress stimulation.

All of the proposed experiments will be carried out in mice, rats, or guinea pigs. All of the studies will be performed utilizing mice of either C3H strain, C57/BL6J strain, DBA-2 strain, and CF-1S strain. Male as well as female mice will be utilized to assess sex as well as strain differences. Rats (Sprague-Dawley and Long-Evans will be used in those studies concerned with cardiovascular tissue; where appropriate to consider strain differences, mice will be used in such studies. The studies on gastric tissue will be carried out in mice, rats and guinea pigs; the latter are particularly susceptible to stress-induced gastric ulceration and the tissues of various organs show an increase in 5-HT concentration upon acute exposure to cigarette smoke.

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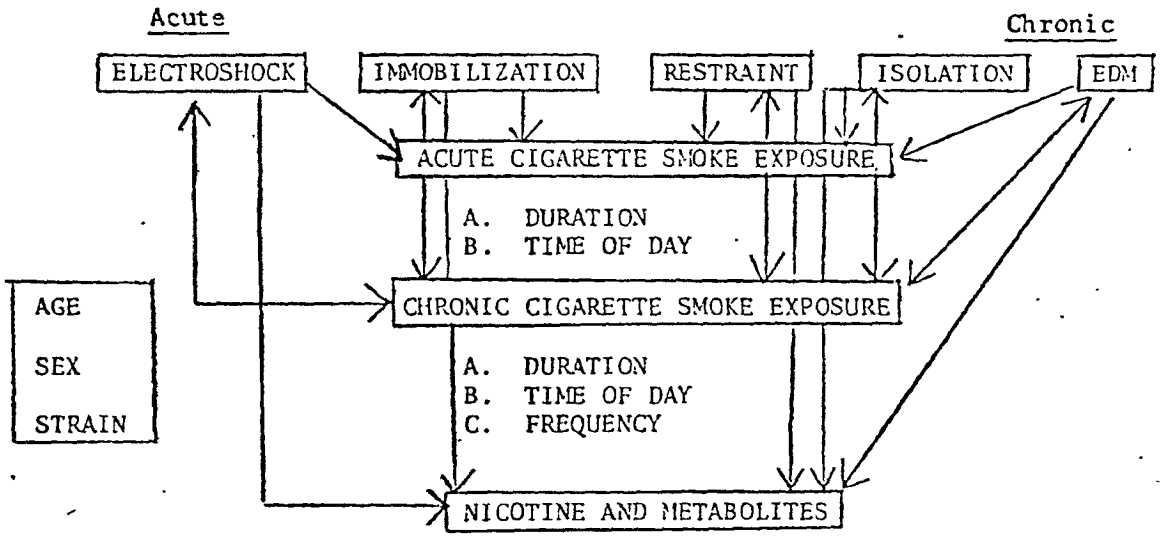
STRESS TOBACCO SMOKE INTERACTIONS:
INDEPENDENT VARIABLES AND SITES OF EFFECT



BRAIN

1. REGIONAL
   - CEREBRAL CORTEX
   - BASAL GANGLIA
   - DIENCEPHALON
   - LIMBIC REGION
   - CEREBELLUM
2. CELLULAR
   - NUERONS
   - GLIA
   - SYNAPTOSOMES
3. SUBCELLULAR
   - SYN. VESICLES
   - MEMBRANES
   - NUCLEI
   - MITOCHONDRIA
   - MICROSOMES

LUNG

1. EPITHELIAL TISSUE
2. PULM. ALVEOLAR MACROPHAGES
3. CELLULAR
   - SEPTAL CELLS
   - EPITHELIAL CELLS
   - KULSCHITSKY CELLS

HEART

1. REGIONAL
   - PERICARDIUM
   - MYOCARDIUM
2. CELLULAR
   - ENDOTHELIAL
   - PURKINUE
   - MYOC. FIBERS
3. SUBCELLULAR
   - NUCLEI
   - MITOCHONDRIA
   - MICROSOMES

G.I. TRACT

1. REGIONAL
   - FUNDUS
   - BODY
   - ANTRUM
   - PYLORIS
2. CELLULAR
   - MUCOSAL
   - SUBMUCOSAL
   - MUSCULARIS
   - ENTEROCHROMAFFIN
   - MAST

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10. Space and facilities available (when elsewhere than item 2 indicates, state location). Two large (approximately 800 square ft.) air-conditioned laboratories are completely equipped with all of the service facilities and permanent equipment necessary to carry out the proposed projects. Both laboratories are provided with ample space to accommodate the principal investigator and the laboratory technical staff. A separate office facility for the principal investigator and the grant project secretary is in use and this contains all of the necessary equipment (i.e., electric typewriter, APECO Copy Machine, etc.) to carry out the administrative portion of the grant research. Additional separate facilities include a vivarium in which separate rooms have been assigned specifically to the principal investigator for any special housing requirements necessary for rodents required as outlined in the grant proposal. Additionally, a histology laboratory with a full-time histologist, machine shop facility, with two full-time machinists, photograph laboratory with a full-time photographer, and electronics facility, with a full-time electronics technician, are all available to the principal investigator in connection with any specific requirements that the research protocol necessitates.

Basic equipment available in our existing research laboratories that will be specifically utilized in the conduct of the research proposed include a Beckman Ultra-Centrifuge, Sorvall High Speed Centrifuge, 3 Sorvall Table-top centrifuges, a Beckman DBG Spectrophotometer, Bausch & Lomb Spectrophotometer, Spectronic 20 Colorimeter, Farrand Spectrofluorometer, two Farrand Filter Fluorometers, 4 refrigerators, 3 freezers, a Kontes Glass Water Distillation Apparatus, and basic laboratory glassware and hardware. A Joelco Scanning Electron Microscope is available for use on a time-available basis.

11. Additional facilities required:

None

12 Biographical sketches of investigator(s) and other professional personnel (append)

Appended are biographical sketches of Dr. Walter B. Essman and Barbara Kornreich.

13. Publications (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached publication list.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Walter B. Essman, M.D., Ph.D. Principal Investigator Sabbatical Leave Supplementation* from 9-1-76 to 12-31-76	100%	
Summer - 2/9ths	100%	REDACTED
Barbara Kornreich	100%	
Technical		
Nancy Mulligan (Secretary)	100%	REDACTED
Richard Rosenthal (Assistant Technician)	100%	
Plus 20% Fringe Benefits		
Sub-Total for A		

B. Consumable supplies (by major categories)

Chemicals	850.
Radiochemicals	1,500.
Centrifuge tubes, supplies	900.
Gases	400.
Glassware	780.
Animals	775.
Sub-Total for B	$5,205.

C. Other expenses (itemize) (Travel)

One domestic meeting and one international congress plus local travel	2,000.
Sub-Total for C	2,000.
Running Total of A + B + C	REDACTED

D. Permanent equipment (itemize)

NONE

Sub-Total for D	-----
E	$ 9,297.
Total request	$71,274.

*50% is requested for the first four months of the 76/77 Academic Year. 50% salary will be provided by the College for Sabbatical Leave. The Principal Investigator will devote 100% time to the research.

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED REDACTED	$5,205.00	$2,000.	------	$10,379.53	REDACTED
Year 3		$5,205.00	$2,000.	------	$ 8,700.42	

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Metabolic Response to Stress - Tobacco Smoke Interactions"	RF-09267 C.T.R. #836A	$61,254.	1/1/75 to 12/31/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Walter B. Essman

Signature Walter B. Essman Date June 30, 1975

Telephone (212) 520-7619
Area Code Number Extension

Checks payable to

Research Foundation of C.U.N.Y.

Mailing address for checks

Mr. Paul Segall, Comptroller
Research Foundation of C.U.N.Y.
1411 Broadway
New York, New York 10018

Responsible officer of institution

Typed Name Hannah Petzenbaum, Res. Foundation Queens College.

Title

Signature David H. Spiedel Queens College Assoc. Dean of Faculty, Date 6/12/75

Telephone 6/12/75
Number Extension

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R: REDACTED MATERIAL

Biographical Sketch

Name: Walter B. Essman

Birthdate & Place: REDACTED

Citizenship: REDACTED

Social Security #: REDACTED

Education:
New York University, B.A.
University of North Dakota, M.A.
University of North Dakota, Ph.D.
University of Milan, M.D.

Post-Doctoral Training:
Senior Post-Doctoral Fellow (Neurophysiology)
Albert Einstein College of Medicine, Bronx, N. Y. 1959-1961.

Fellow, Dept. of Neurology (Neurochemistry)
Mt. Sinai Hospital, New York, N.Y. 1964-1968

Clinical Training: Rotating Internship and Residency in Internal Medicine

Licensure in Medicine:
New York State
State of Maine REDACTED

Military Service: U.S. Army, 1957-1957, Captain, MSC.

Academic Experience:
Professor (Psychology, Biochemistry), Queens College of the City University of New York, 1967 - Present.

Lecturer (Medicine), State University of N.Y. at Stony Brook, School of Medicine, 1974 - Present.

Associate Professor, Queens College of the City University of New York, 1965 - 1966.

Associate Professor, Queens College of the City University of New York, 1962 - 1964.

Research Professor, Neurology, Mt. Sinai School of Medicine New York, 1972 - 1974.

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Research Associate, Laboratory of Neurochemistry, Mt. Sinai School of Medicine, New York, 1966 - 1972

Research Assistant Professor, Department of Rehabilitation Medicine, Albert Einstein College of Medicine, 1962 - 1963.

Research Associate, Dept. of Physiology, Albert Einstein College of Medicine, 1961 - 1963.

Director, Psychophysiological Research, U.S. Army Surgical Research Unit, 1958 - 1959.

Membership in Professional Societies:

REDACTED

REDACTED

Other Professional Activities:

Associate Editor, Psychological Reports, 1959 - Present
Advisory Editor, Communications in Behavior
Consulting Editor, Physiology and Behavior, Journal of Neurobiology
Editor, Series in Modern Neurobiology, Spectrum Publications
Editor, Current Developments in Psycho . rmacology, Spectrum Publications, N.Y.

Honors and Awards:

Sigma Xi, 1957
Phi Delta Kappa, 1957
Ambrogino d'Oro - Municipality of Milan, 1971

Directory Listings:

Who's Who in Science
Who's Who in the East
American Men of Science
Outstanding Educators of America
Dictionary of International Biography

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R: REDACTED MATERIAL

Biographical Sketch

Name: Barbara Saplin Kornreich

Birthdate & Place: REDACTED

Citizenship: REDACTED

Social Security #: REDACTED

Education: State University of N.Y. at Albany, 1964, B.A.

Employment Experience:

New York Department of Health, Albany, N.Y., Assistant to post-doctoral fellow, Dr. M. Z. Atassi, 1963 - 1964

State University of New York at Buffalo, Sr. Research Assistant for Dr. M. Z. Atassi, Depts. of Biochemistry and Oral Biology, Schools of Medicine and Dentistry Duties: Protein research involving column chromatography, electrophoresis, Amino Acid analysis and immunochemical characterization techniques, 1964 - 1967.

Long Island Jewish Medical Center, New Hyde Park, N.Y. Sr. Research Associate to Dr. Morton Urivetzky. Duties: General supervision of laboratory operations; carried out research on collagen. All general procedures were used including the use of radio-immunoassay. 1967 - 1971.

Queens College of the City University of New York. Research Assistant to Dr. Walter B. Essman, Depts. of Psychology and Biochemistry: Research involves work with brain serotonin. 1973 - Present.

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Publications

1972

1. Essman, W.B.: Effetti Dell'Elettroshock Sulla Neurochimica Del Sistema Nervaso Centrale, I. Rass. Clin. Scient: 48: 361-370.

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2. Essman, W.B.: Effetti Dell'Elettroshock Sulla Neurochimica Del Sistema Nervaso Centrale, II. Rass. Clin. Scient: 49: 5-23.

3. Essman, W.B.: "Subcellular Actions of Benzodiazepine Action". In Garattini, S., Mussini, E. and Randall, L. O. (Eds.): The Benzodiazepines, New York: Raven Press, Pp. 177-190.

4. Essman, W.B.: Neuromolecular Modulation of Experimentally Induced Retrograde Amnesia. Confinia Neurol: 35: 1-22.

5. Essman, W.B.: "Nicotine-Related Neurochemical Changes: Some Implications for Motivational Mechanisms and Differences." In Dunn, W.J., Jr. (Ed.): Smoking Behavior, Pp. 51-65.

6. Essman, W.B.: Age-Dependent Effects of 5-Htdroxytryptamine upon Memory Consolidation and Protein Synthesis. Pharmacol. Biochem. and Behavior. 1: 7-14

7. Essman, W.B. and Essman, S.G. Biphasic Effects of Nicotine on ECS-Induced Retrograde Amnesia in Mice. Psychol. Rep.: 32: 658.

8. Essman, W.B.: Tissue Distribution and Central Effects of Digoxin in Mice: Effects of an Acute and Chronic Stress. Pharm. Res. Commun. 5: 303-310.

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9. Essman, W.B.: "Brain 5-Hydroxytryptamine and Memory Consolidation." In: Costa, E., Gessa, G.L. and Sandler, M. Advances in Biochemical Psychopharmacology, 11: Serotonin: New Vistas. New York: Raven Press, Pp. 265-274.

10. Essman, W.B.: Regional Alterations of Synaptic O-Phosphorylethanolamine in Differentially-Housed Mice. Pharm. Res. Commun: 6: 377-395.

11. Essman, W.B.: "Amine Mediated Retrograde Amnesia: Factors Effecting Cerebral Protein Synthesis". In Bogoch, S. (Ed.): Biological Diagnosis of Brain Disorders. New York: Spectrum Publications, Pp. 165-175.

12. Essman, W.B.: "Effects of ECS on Cerebral Protein Synthesis". In Fink, M., Kety, S.S., McGaugh, J., and Williams, T. (Ed.): The Psychobiology of Convulsive Therapy. Washington, D.C.: V.H. Winston & Sons, Pp. 237 - 249.

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13. Essman, W.B.: Diurnal Differences in Altered Brain 5-Hydroxytryptamine-Related Regional Protein Synthesis. Journal de Pharmacologie, In Press.

14. Essman, W.B. "Biochemical Plasticity Among Differentially Housed Mice". In: Boissier, J.R. (Ed.). Neurospychopharmacology, Amsterdam: Excerpta Medica, In Press.

15. Essman, W. B., Kimmelstiel, F. & Sporer, B. Age-Related Determinants of Stress-Induced Weight Change in Mice. Psychological Reports, 36: 920-922.

16. Essman, W.B., Abrams, R., Taylor, Michael A.and Fink, M. Concentration of 5-Hydroxyindoleacetic Acid, Homovanillic Acid and Tryptophan in the Cerebrospinal Fluid of Depressed Patients Before and After ECT. Biol. Psychiat., In Press.

Abstracts

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2. Essman, W.B. and Heldman, E.: Effect of 5-Hydroxytryptamine (5-HT) on Cerebral Protein Synthesis: In Vivo Mediation and In Vitro Effects. Abstr. Soc. for Neuroscience, 3rd Meeting, San Diego, Calif., 1973, 327, 56.2

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METABOLIC RESPONSE TO STRESS -

TOBACCO SMOKE INTERACTIONS

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Summary of Progress

For approximately 2½ years our laboratory has been studying stress-tobacco smoke interactions at a molecular metabolic level. Experiments were concerned with either acute (electroshock, immobilization) or chronic (isolation housing) stress. In the mouse, these have been quantified for their behavioral (aggressive behavior), physiological (sleeping time, diurnal activity), pharmacological (tissue distribution of drugs), and biochemical (amine uptake, protein synthesis) effects at various tissue sites. The interaction of stress with either single or multiple exposure to standardized cigarette smoke or to physiological concentrations of nicotine was studied in a number of experiments.

The concentration of acetylcholine, a neurotransmitter, was decreased by 50% or more following stress (Essman, 1972). The central nervous system was particularly vulnerable to the effects of both acute and chronic stress. Electroconvulsive shock led to increases in 5-hydroxytryptamine concentration (17%) and in its metabolism (increased turnover); this effect was further augmented in chronically stressed mice (See Table 1) (Essman, 1973 (2)).

Table 1

Per Cent Concentration Change in Regional 5-Hydroxytryptamine Concentration by Electroconvulsive Shock in Chronically Stressed (Isolation) and Control Mice

BRAIN REGION	CHRONIC STRESS	NO STRESS
Olfactory Bulbs	42*	12.5
Cerebral Cortex	22*	9.5
Mesencephalon	7	21*
Diencephalon	5	23*
Cerebellum	0	25

*p<.02

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A relationship between isolation stress, altered brain 5-HT metabolism, and the regulation of brain microsomal protein synthesis was shown (Essman, 1975 (13)). Increased brain 5-HT concentration led to reduced rates of protein synthesis. Nicotine (0.7mg/kg) led to a 59% increase in the rate of microsomal protein synthesis in the cerebral cortex. Changes in brain acetylcholine (ACh) content in both acute and chronic stress were described in similar studies (Essman, 1971). Nicotine produced a reduction in vesicular (52%) and bound (80%) ACh. In stressed mice nicotine uptake into several regions of the brain was significantly increased; i.e., cerebral cortex (96%), hippocampus (43%).

Diurnal changes in brain 5-HT concentration were also related to brain protein synthesis; when endogenous levels of brain 5-HT were high (early AM), the rate of protein synthesis was low; when endogenous levels of this amine were low (PM), high rates of regional protein synthesis were observed (Essman, 1975 (13)). (See Table 2).

Table 2

Diurnal Differences in the
Regional Intercorrelation of ECS or Drug-Induced
Changes in 5-Hydroxytryptamine and Microsomal Protein Synthesis

REGION	AM	PM
Cerebral Cortex	-0.14	-0.74*
Diencephalon	+0.40	+0.76
Limbic Area	-0.94*	+0.56
Cerebellum	+0.67	+0.47

*p .01

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Male mice that were isolated developed aggressive behavior but female mice did not. The effects of chronic stress (isolation) in male mice accellerated gonadal (197%), but not adrenal testosterone synthesis. Stressed female mice, although showing a 135% increase in adrenal lipid synthesis, did not show an increased rate of testosterone synthesis (Essman, et al., 1973 (1)).

During the past year, these basic studies have found application in the investigation of stress determinants in mice exposed to tobacco smoke. Various animal holders have been studied to determine their stress effects. The adrenal response to stress has been shown to occur (by elevation of plasma corticosterone (PC) level) by immobilizing mice for intervals as short as 8 minutes. Interestingly, maximal stress, as suggested by PC levels of approximately 180% above baseline (.11 to .15 µg/ml) occurred by 20 minutes of smoking machine confinement, with no subsequent further increase up to one hour (Essman, et al, 1975 (15)). Tobacco smoke introduced into the machine caused no additional elevation of plasma corticosterone content, suggesting that it did not augment the stress response initiated by immobilization.

It was further of interest to observe that adaptation (successive reduction of PC level to within baseline range) would occur with continued exposure to the smoking machine. Adaptation, as measured by decreased plasma corticosterone levels occurred following three weeks of daily exposure to cigarette smoke (8 minutes) for mice immobilized in a smoking maching. Once more, tobacco smoke had no additional effect upon PC levels.

Another measure of stressor effect was cardiac norepinephrine uptake; this was reduced with restraint stress, consisting of 8 minutes of immobilization (66%). Adaptation to stress (gradually increased rates of cardiac NE uptake to

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within baseline limits) was also observed after four weeks of daily exposure. No additive or potentiated effects occurred with tobacco smoke, except after 21 days or more of repetitive exposure; at this point cigarette smoke exposure interfered with the stress-induced reduction in NE uptake.

It was possible, through the use of cell fractionation methods, to isolate presynaptic nerve endings (synaptosomes) and their constituent synaptic vesicles. The uptake of 5-HT differed for synaptic vesicles from different mouse strains, C3H showing the lowest uptake and DBA showing the highest. Chronic stress (isolation) reduced vesicular 5-HT uptake for mice of the DBA and C57 strains, but did not alter uptake by vesicles from the C3H strain. Tobacco smoke reversed the effects of stress in C57 strain mice; i.e., smoke exposed C57 strain mice did not show a stress-induced reduction in 5-HT by synaptic vesicles.

Regional (cerebral cortex, diencephalon, cerebellum) uptake of amines (5-HT, norepinephrine, and dopamine) was increased in synaptosomes following in vivo amine depletion; the phenomenon of denervation-supersensitivity (Cannon & Rosenbleuth, 1929) was illustrated in these experiments. This principle holds that a reduction of neural innervation results in a reduction in threshold to activation and increased activity in such cells or tissue. Increased amine uptake by intact nerve endings after a reduction in their number is support for increased sensitivity following reduced innervation.

The question of how basic differences in cellular response to nicotine occurred at physiological concentrations was raised. To deal with this issue, a newly developed tissue fractionation method was used to separate neurons and glia from different brain regions.

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In several in vitro studies, it was possible to show that physiological concentrations of nicotine (2.5×10^{-7}M) altered amine uptake by isolated nerve cells and their adjacent glia. Specifically, nicotine increased 5-HT (124%) and dopamine (31%) uptake in diencephalic neurons; in glia from the basal ganglia (30%) and diencephalon (29%) showed increased NE uptake. Decreased dopamine uptake occurred in cortical (76%) and cerebellar (24%) glia; there were no changes in cells of the basal ganglia.

The significance of these findings lies in the regional and cellular specificity with which physiological levels of nicotine act. With amine uptake as an index of decreased receptor availability, one could postulate different regional and cellular sites of excitability change. Cortical and cerebellar excitation may be inferred from the present findings. The absence of any uptake change for cells from the basal ganglia have particular interest since another measure of cellular stimulation is increased protein synthesis -- the basal ganglia represents one region of the brain where the constituent cells show changes in protein synthesis from nicotine.

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Protein synthesis was stimulated by physiological concentrations of nicotine, specifically in neurons (59%) and glia (24%) from the basal ganglia; in other brain regions, there was no difference at this observed concentration of nicotine (10^{-7}M). These findings hold some potential clinical significance for Parkinson's disease. Decreased basal ganglia dopamine levels in this disorder have been associated with reduced protein synthesis. It is possible that nicotine could reverse this effect. This relationship bears further investigation.

Tobacco smoke exposure (8 min.) elevated pulmonary 5-HT concentration for certain strains of mice (C3H, DBA, CF1); pulmonary 5-HT content was not altered by tobacco smoke in mice of the C57 strain. These data have been summarized in Table 3.

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Table 3

Effect of Tobacco Smoke Exposure
Upon 5-Hydroxytryptamine Content of the Pulmonary
Epithelium of Male Mice of Several Inbred Strains

CONDITION	STRAIN		
	CF-1S	DBA-2	C57/BL6J
Tobacco Smoke	0.22* (0.01)	0.18 (0.01)	0.05 (0.01)
Control	0.18 (0.01)	0.11 (0.02)	0.04 (0.01)

*p <.01

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Pulmonary alveolar macrophage yeild was decreased by 32% after 8 minutes of cigarette exposure. Macrophages were shown to bind 5-HT (5.78×10^{-12} Moles); such binding was increased (14.59×10^{-12} Moles) by the gas phase of cigarette smoke, suggesting that the gas phase, in the absence of nicotine increased the number of binding sites on the macrophage. Some strains, such as DBA, yielded macrophages with significantly greater number of binding sites (1.59×10^{-14} Moles), whereas other strains, e.g. C3H, yielded macrophages with an appreciably fewer number of binding sites (0.05×10^{-14} Moles). Tobacco smoke exposure significantly ($p<.01$) decreased 5-HT binding by macrophages (79%) in DBA strain mice, but in no other strain. Stress (isolation) decreased binding in C57 (62%) and DBA strains, but not in C3H mice. Tobacco smoke exposure blocked stress-induced reduction in macrophage binding of 5-HT in C57 mice. These studies suggest strain specificity for (1) macrophage yield, (2) macrophage yield following acute exposure to cigarette smoke, (3) macrophage binding of 5-HT, (4) stress-induced alteration in macriphage binding, and (5) interaction of stress with tobacco smoke in

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determining macrophage binding. This appears to be the first report of chronic stress affecting macrophage binding capacity. The ability of tobacco smoke to alter this effect, at least in one strain of mouse, indicates a need for further investigation.

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Psychological Reports, 1975, 37, 112-114. © Psychological Reports 1975

AGE-RELATED DETERMINANTS OF STRESS-INDUCED WEIGHT CHANGE IN MICE[1]

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Summary.—25-day-old male mice showed a greater incidence of body weight loss and a late peak of plasma corticosterone elevation when exposed to tobacco smoke for 5 successive days, as compared with mice exposed to filtered smoke (gas phase) or air. 50-day-old mice had a greater incidence and magnitude of body weight loss and a late plasma corticosterone peak after 5 days of air exposure, but not with gas phase or nicotine + gas phase. Diencephalic serotonin, while higher among 25-day-old mice, did not differ as a function of the stress of confinement during exposure to the ventillatory stimulus or as a function of the different stimuli. The results suggest that weight loss and the time following stimulation at which plasma corticosterone is maximally elevated may serve as indices of stress and are consistent even when the stressor-susceptibility is age-related.

The acute exposure of mice to cigarette smoke and/or its biologically active ingredients has been shown to effect changes that relate in several ways to the mediation of stress effects (Selye, 1973). Such relationships, in particular, concern central regulation of ACTH release and the adrenal secretion of corticosterone but more generally concern anabolic measures such as change in body weight. There is good basis for relating weight change both to the age at which it occurs and also to stress-related factors that may affect it as a function of age. The concern of the present study was to investigate the interaction of a stressor event—physical restraint, with exposure to cigarette smoke delivered during five successive daily trials.

A total of 120 male CF-1S strain mice were randomly assigned to groups in which each animal received five daily (8 a.m. to 9 a.m.) exposures to either air, cigarette smoke, or filtered cigarette smoke (delivering the gas phase only with no nicotine or metabolites) for 24 min. in a Walton Upright Smoking Machine. Half of the mice were 25 days old and the others 50 days of age at the start of the study.

Mice were weighed at the beginning and end of the study and at intervals of 15, 30, 45, or 60 min. following the fifth daily exposure, they were killed by cervical dislocation; samples of blood were taken from the abdominal aorta and brain tissue was removed. The plasma was assayed for corticosterone (Zenker & Bernstein, 1958) and the brain tissue was regionally subdivided and assayed for serotonin and 5-hydroxyindoleacetic acid (Welch & Welch, 1969).

Weight differences between mice within any group could not be accounted

[1]This study was supported in part by a grant from the Council for Tobacco Research, U.S.A.

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4

NICOTINE-RELATED NEUROCHEMICAL CHANGES: SOME IMPLICATIONS FOR MOTIVATIONAL MECHANISMS AND DIFFERENCES[1]

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Some of the possible ways in which the pharmacological role of nicotine underlies the smoking habit have been reviewed by other investigators (Murphree, 1967; Larson and Silvette, 1968; Jarvik, 1970). The favored view holds that the motivational relevance of nicotine resides in its effects upon the central nervous system (Murphree, 1967). For the most part these effects have been resolved with a central catecholamine hypothesis (Burn, 1960). This chapter will redirect attention to the fact that, either directly or indirectly, nicotine also induces other biochemical changes in the brain, changes which could as well have motivational significance.

Work already reported by this writer has related changes in the motivated behavior of experimental animals to (*a*) changes in the concentration and turnover of 5-hydroxytryptamine (Essman, 1969), and to (*b*) changes in the central cholinergic system (Essman, 1971a). The relevance of the effects of nicotine to these reported relationships is to be found in the observation that nicotine induces changes in both the central indoleamine and cholinergic systems (Essman, Golod, & Steinberg, 1968; Essman, 1971a). These findings and the meagerness of evidence to support the central catecholamine effect of nicotine (Westfall, Flemming, Fudger, & Clark, 1967), suggest that any attempt to investigate motivation in human smoking should include a study focused upon the effects of nicotine upon the central indoleamine and cholinergic systems.

In order to study the central nervous system effects of nicotine, we have chosen 5-hydroxytryptamine (5-HT) and acetylcholine (ACh)—considered on a regional, cellular, and subcellular level. We have also investigated the possibility that differences in central amine level and metabolism are related to differences in the

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18

EFFECTS OF ELECTROCONVULSIVE SHOCK ON CEREBRAL PROTEIN SYNTHESIS[1]

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In previous studies I have examined the relationship between electroconvulsive shock as a vehicle through which retrograde amnesia may be provided in experimental animals and several biochemical alterations that have been observed as a consequence of such treatment. Utilizing a single transcorneal ECS, rather consistent biochemical changes in mouse forebrain have been observed; these included: (*a*), a decrease in the regional concentration of ribonucleic acid (RNA), with such a reduction dependent upon the age at which ECS treatment has been given (Essman, 1966, 1972); (*b*), a reduction in the incorporation of precursors into brain RNA, which has also been shown to be age-dependent (Essman, 1971); (*c*), an increase in forebrain and regional levels of 5-hydroxytryptamine (5-HT) observed shortly following a single ECS (Essman, 1967, 1968, 1970b); (*d*), an increase in ECS-induced brain 5-HT turnover time (Essman, 1968, 1970a); and (*e*), a relationship between RNA, free nucleotides, and 5-HT in brain that is altered as a consequence of cerebral electroshock (Essman, Bittman, & Heldman, 1971; Essman, 1971); e.g., electroshock has also been shown to produce changes in those cerebral nucleotides which are related to excitability functions (Bonavita & Piccoli, 1971).

In view of several of the previous relationships demonstrated between ECS and several endogenous constituents in brain probably relevant to regulatory mechanisms operative during cerebral protein synthesis, it has been my purpose to consider some of those parameters by which the effects of ECS on protein

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[2]The author wishes to acknowledge the technical assistance rendered by Mr. E. Heldman for some of the reported experiments.

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The Benzodiazepines, Raven Press, New York © 1973

SUBCELLULAR ACTIONS OF BENZODIAZEPINES

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Cell Fractionation Methodology

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I. INTRODUCTION

The central action of benzodiazepines has been given limited selective review (Randall, 1961, 1968) regarding their pharmacological properties and behavioral effects, but few consistent data have been provided concerning the effects of these compounds upon central nervous system metabolism. One of the several problems encountered in the study of the central action of benzodiazepines concerns the relationship between the uptake and deposition of such molecules and the specific locus where their central effects occur and through which their functions are modulated. This series of studies investigated several neurochemical changes associated with the action of two benzodiazepine compounds. Previous behavioral indications and studies of time course for central uptake, following parenteral administration, served as a basis for consideration of their action in terms of central synaptic localization and function.

Preliminary investigations in our laboratory suggested that the guinea pig was maximally sedated 60 min following the parenteral injection of chlordiazepoxide (CDP) in doses up to 10 mg/kg. It was further established that at these times maximum drug recovery (as measured from brain tissue by low-temperature spectrofluorescence assay following heptane-alcohol and ethyl acetate extraction from regional brain-tissue homogenates) was from the cerebral cortex, which represents a substantial proportion of the total brain weight of the guinea pig. On the basis of these preliminary findings and with the operating premise that CDP and related benzodiazepines probably exert profound central synaptic actions in bringing about their other physiological and behavioral effects, several indexes of such cerebral synaptic function were utilized as measures of CDP action, including electrolyte concentration and distribution, ribonucleic acid (RNA) content, and acetylcholine (ACh) levels.

Specific concern was given to the effects of two benzodiazepine compounds, CDP and SCH 12041, upon synaptosomal elements and subcellular

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GROWTH HORMONE AND SEROTONIN ANTAGONIST

To the Editor: In their recent paper Bivens, Lebovitz, and Feldman[1] presented data showing attenuation of the response of serum growth hormone (GH) to insulin stimulation in normal subjects treated with cyproheptadine or methysergide. Because an increase in hypothalamic serotonin content has been found in laboratory animals during induced hypoglycemia, and because intraventricular administration of serotonin stimulates GH secretion in the rat, the authors concluded from their experiment that these two serotonin antagonists probably interfered with hypothalamic serotonin stimulation of GH secretion. Most pharmacologic studies, however, would argue otherwise.

The antiserotonin activity of cyproheptadine and methysergide appears to be confined to peripheral receptor-site blockade of this monoamine.[2] Neither drug changes central-nervous-system serotonin concentration nor has central antiserotonin action at doses appreciably below the LD_{50} for laboratory animals.[3,4] Indeed, because it is strongly hydrophilic, methysergide is poorly transported across the blood-brain barrier, if at all.[5] It therefore seems unlikely that these agents act centrally to modify pituitary secretion of GH. How they act remains unknown. Possibly, they reduce serum GH by affecting the hormone's peripheral metabolism.

It is sometimes as easy to tell which comes first in the serotonin-GH relation as it is in the chicken-egg one. For example, in animals treated with GH there is increased weight and granularity of enterochromaffin cells,[6] and increased urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA),[7] whereas opposite effects on the enterochromaffin cells are noted after hypophysectomy.[8] Thus, an increase in serum GH may stimulate serotonin synthesis and turnover in peripheral serotonin-rich pools. We have some data to suggest that serum GH affects serum serotonin concentration in man. Five acromegalic patients had a mean fasting serum GH of 27.90 ± 6.31 μg per milliliter and elevated mean fasting serum serotonin level of 0.91 ± 0.31 μg per milliliter. Within three months after trans-sphenoidal resection of the pituitary tumor in this group, mean serum GH level fallen to 7.62 ± 2.33, and mean serum serotonin to 0.57 ± 0.10 μg per milliliter. The serum serotonin–5-HIAA ratio was [illegible] high (5.8:1) before and was normal (2.1:1) after operation—a statistically significant decline (chi-square = 17.02, $p < 0.01$). Thus, peripheral blood levels of serotonin appeared to follow changes in serum GH produced by pituitary-adenoma resection. Whether the reverse effects (changes in peripheral serotonin concentration, or in the ability of serotonin to attach to appropriate peripheral receptor sites) induce changes in serum GH are unknown to us.

Though we question the presumed mechanism, we applaud the discovery of attenuation of serum GH response to hypoglycemia by serotonin antagonists. These agents may be useful, possibly when combined with compounds having central antiadrenergic activity,[9] in experimental drug therapy of acromegaly and gigantism.[10]

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Essman, W.B., Kostowski, W. and Valzelli, L.: Alterations in Aggressive Behavior in Mice with Changes in 5-Hydroxytryptamine Regulation. Fed. Proc., 33: 1974, 446.

ALTERATIONS IN AGGRESSIVE BEHAVIOR IN MICE WITH CHANGES IN BRAIN 5-HYDROXYTRYPTAMINE REGULATION. W.B. Essman, W. Kostowski* and L. Valzelli*. Queens College of the C.U.N.Y., Flushing, N.Y. 11367 and Mario Negri Inst., Milan, Italy.

The modulation of aggressive behavior resulting from isolation housing in mice was investigated in several studies in which 5-hydroxytryptamine (5-HT) regulation was altered. Male albino mice, isolation housed for 28 days, showed somewhat elevated 5-HT levels (14%) and a 2.7 fold increase in 5-hydroxyindoleacetic acid (5-HIAA) concentration. When 5-HT synthesis was interrupted by inhibition of tryptophane hydroxylase with p-chlorophenylalanine (PCPA)--300 mg/kg at 3-day intervals, and brain 5-HT levels were reduced (0.78 ± 0.11 to 0.47 ± 0.12) during isolation housing, aggressive behavior was reduced (50%). Group-housed mice given comparable PCPA treatment became aggressive after 4 injections. The aggressive behavior in isolated mice was completely abolished when measured 3 days or more after lesions of the nucleus magnus of the ventral raphe; brain 5-HT level was reduced (0.64 ± .03 to 0.38 ± .02 µg/g; $p<.001$) as was 5-HIAA (0.32 ± .02 to 0.17 ± .01; $p<.001$). Decreased brain 5-HT in PCPA-treated grouped mice increased microsomal protein synthesis in cerebral cortex (46%; $p<.01$); and diencephalon (13%; $p<.05$); such protein synthesis in PCPA-treated isolated mice was inhibited in the limbic system (13%; $p<.05$). Attenuation of isolation-induced aggressive behavior has been related to both alteration in 5-HT regulation of protein synthesis and metabolism. (Supported in part by a Grant from the Council for Tobacco Research, U.S.A.

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Essman, W.B. Biochemical Plasticity Among Differentially Housed Mice. J. Pharmacol. (Paris), 5: 1974, 92-93.

BIOCHEMICAL PLASTICITY AMONG DIFFERENTIALLY HOUSED MICE. W.B. Essman, Queens College of the C.U.N.Y., Flushing, New York (U.S.A.)

Attending several behavioral changes among different inbred strains of mice, housed either in isolation or in groups, such as avoidance acquisition deficits, aggressive behaviors, and changes in sleep behavior and activation, endogenously or through the effects of psychoactive drugs, are numerous alterations in neurochemical parameters of functional status. Isolation provided for a decrease in the RNA content of cerebellar neurons (34.1 to 19.50 µg/cell), whereas glial RNA content was increased (45.2 to 78.3 µg/cell). Synaptosomal RNA from cerebral cortex was markedly decreased as a consequence of isolation (10.8 to 1.9 µg/mg protein), and there were comparable reductions in various organelle fractions derived from such nerve endings. 5-HT uptake by cerebral cortex nerve endings was significantly reduced (64%) and non-linear with concentration as a consequence of isolation. Isolation housing led to an increase in cerebral lipid synthesis (31%) with decreased lipid synthesis in liver (59%) and unchanged synthesis in kidney. ECS, while significantly increasing brain lipid synthesis in both isolated (4.0 X) and grouped (5.6 X) mice did not appreciably affect synthesis by liver or kidney. Receptor antagonism of 5-HT with methysergide (1.0 gm/kg) potentiated isolation-induced increases in brain lipid synthesis (21%), as did scopolamine (1.3 mg/kg - 61%); however, benzodiazepines (25 mg/kg) did not alter this effect of isolation.

Incubation of isolated microsomal fractions from cerebral and cerebellar cortex of isolated and grouped mice in the presence of 5-HT led to significant inhibition of C^{14}-leucine incorporation into microsomal proteins (25% and 94% respectively) in tissues from grouped mice. Among isolated animals, the respective effects of 5-HT were significantly reduced to 8 and 33%, respectively.

Isolated male mice showed increased synaptosomal uptake of 5-HT in the cerebral cortex (33%) and cerebellar cortex (14%), whereas 5-HT uptake by synaptosomes in female mice was not significantly altered by isolation.

Isolated mice had significantly ($p<.01$) decreased activity of acetylcholinesterase (AChE) in the frontal (71%), parietal (56%), temporal (63%), and occipital (59%) cortex; however, environmental stimulation (tactile, auditory, visual, and vestibular) for 7 days altered AChE activity. Grouped mice showed significant decreases in enzyme activity in frontal (49%), parietal (41%), temporal (50%), and occipital (46%) cortex, whereas stimulated isolated mice showed significantly increased AChE activity in frontal (150%) and temporal (52%) cortex; enzyme activity in these regions was now comparable to that of non-stimulated grouped mice and exceeded that of stimulated grouped animals. The subsynaptic distribution of AChE in fractions from the cerebral cortex was also modified by isolation. Enzyme activity associated with small external synaptic membrane fragments (MFα) was significantly reduced, as compared with that obtained from grouped conditions (0.15 A.U./mg/min. to 0.03 A.U./mg/min.). Isolation provided for increased synaptosomal acetylcholine (ACh) levels (400 ± 0.93) as compared with grouped animals (2.40 ± 0.72 µMoles/g).

The plasticity of CNS biochemical alteration resulting from isolation housing appears important for the behavioral and pharmacological changes for which such differential housing provides.

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

Essman, W.B. Diurnal Differences in Altered Brain 5-Hydroxytryptamine-Related Regional Protein Synthesis. J. Pharmacol. (Paris), 5: 1974, 28.

DIURNAL DIFFERENCES IN ALTERED BRAIN 5-HYDROXYTRYPTAMINE-RELATED REGIONAL PROTEIN SYNTHESIS. W. Essman, Queens College of the C.U.N.Y., Flushing, N.Y. (U.S.A.)

The relationship between regionally altered brain 5-HT level and microsomal protein synthesis was studied at two divergent, diurnal points in the male CF-1S strain mouse. 5-HT was endogenously higher (33 to 60%) in all regions at 8 AM; there was significantly less C^{14}-leucine incorporation into microsomal protein than at 8 PM. 5-HT elevation after electroconvulsive shock was greater in the PM, particularly in the limbic system; PM protein synthesis was inhibited 25% more by ECS than it was in the AM. A significant elevation of 5-HT in all brain regions (58 to 744%) occurred 45 minutes after a single dose of 6-methyl-tetrahydro-3-carboline (50 mg/kg). Increments were greater in the PM in cerebral cortex, but greater AM increments occurred in the limbic system and cerebellum with comparable AM and PM reductions in protein synthesis. p-chlorophenylalanine (PCPA, 300 mg/kg) resulted in decreased 5-HT content, more extensive in the PM in diencephalon (8 times) and limbic system (1.25 times). Increased microsomal protein synthesis occurred after PCPA treatment and this was greater (40%) when the drug was given in the AM. The most significant effect of 5-HT level and microsomal protein synthesis occurred in the cerebral cortex (ρ=-74) and the limbic system (ρ=-94) in the AM when the former were endogenously higher and there was increased regional susceptibility to alterations effected by agents or events which also modified such macromolecular synthesis.

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REGIONAL ALTERATIONS OF SYNAPTIC O-PHOSPHORYLETHANOLAMINE IN DIFFERENTIALLY HOUSED MICE[1]

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SUMMARY

In three experiments, the relationship between brain O-phosphorylethanolamine (PE) and isolation-induced behavioral effects was considered. It was observed that following eight weeks of isolation, male CF-1S strain mice showed highly significant elevations in synaptosomal PE in structures of the limbic system and in the cerebellar cortex; such changes did not occur in the cerebral cortex. Elevation of brain PE in mice by exogenous means led to profound rapid changes in the neurological status of the animal, with a subsequent behavioral status resembling that brought about by isolation housing. A PE-mediated inhibition of cerebral protein synthesis was demonstrated *in vitro* for microsomal fractions, particularly for those derived from the cerebellar cortex. The present data have been considered with a view toward introducing PE as a potential link to inhibitory changes in the central nervous system.

INTRODUCTION

A relationship between neurochemical changes and specific behaviors has been sought in several studies wherein differential housing has been the independent variable (Bennett, *et. al.*, 1964; Valzelli, 1967; Essman, 1969).

[1]This study was supported in part by a grant from the Council for Tobacco Research - U.S.A., and a grant #5-S05-FR-07064-05 from the U.S. Public Health Service.

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#1029-MCKENNIS

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

May 1, 1975

Grant application No. 1029

PHARMACOLOGY

TO: The committee comprising Drs. Bing, Jacobson and Sommers

SUBJECT: Herbert McKennis, Jr., Ph.D., Medical College of Virginia, Richmond, Virginia
New application No. 1029
"Transport and Metabolism of Amine Constituents of Cigarette Smoke"

History

This application was not considered as a case. Dr. McKennis has held CTR grants (under various titles) since 1956. Grant application No. 868R1 "Biological Activity of Tobacco Smoke Components and Allied Substances" was approved in October 1973 as a terminal grant at a reduced level. A new application (No. 970) requesting $38,662 "Pharmacodynamics of Cotinine," the possible blocking of nicotine action at various receptor sites by direct competition with metabolites of cotinine, was denied by the SAB in October 1974.

The emphasis in the enclosed proposal is concerned with the disposition and elimination from the body of a variety of nitrogen bases derived from tobacco smoke.

Request

Application No. 1029 requests $40,396 plus two additional years.

Documents Submitted

1. Application dated April 15, 1975, 15 pages, including CV^{s} of Drs. McKennis, E. R. Bowman and A. F. Bowman and K. L. Wilson, Jr.

2. Two abstracts (1 page each) and a manuscript (39 pages) submitted for publication.

D.S.

DS/lp
Encl.

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1029

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

APR 2 8 1975

Application for Research Grant
(Use extra pages as needed)

Date: April 15, 1975

1. Principal Investigator (give title and degrees):

Herbert McKennis, Jr., Ph.D.
Professor of Pharmacology

2. Institution & address:

Medical College of Virginia
Richmond, Virginia 23293

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology
(Division of Biochemical Pharmacology)
Medical College of Virginia, Richmond, Virginia 23298

4. Short title of study:

Transport and Metabolism of Amine Constituents of Cigarette Smoke

5. Proposed starting date: 1 June 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Various investigations of cigarette smoke have provided evidence for several hundred amine constituents which are absorbed, excreted, and metabolized to varying degrees by man and other animals. Common sense alone would serve to suggest that most of these substances are excreted in one form or another -- as opposed to being permanently and completely stored. Recent reports (Russell and Feyerabend, Lancet, 179 (1975); Falkman et al., Analyst, 100, 99 (1975)), emphasize more and more that significant detectable quantities of nitrogen bases from cigarette smoke are determinable by physical and chemical means in the blood of non-smokers as well as the blood of smokers. The nitrogen bases in cigarette smoke can be considered to range from the simplest ammonia to bases with more complex structure such as harmane and norharmane. Since claims have been made that many of the nitrogenous bases are directly or indirectly involved in the development of pathological conditions in man and animals, factors that determine the disposition of these nitrogen bases become increasingly important. Although pH effects on the urinary excretion of nicotine and a few related compounds have been reexamined in recent years by Beckett et al., various factors such as protein binding, renal tubular excretion and absorption, competition and renal metabolic changes, which are important to the elimination of the nitrogen bases are not well understood or even crudely defined for many important nitrogen bases that are derived directly or indirectly from tobacco smoke. A

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Number 7 continued:

list of some of specific substances where substantial data is required would include cotinine, cotinine-N-oxide, 4-(3-pyridyl)-4-methylamino-butyric acid, demethylcotinine, cotinine methonium ion, allohydrocotinine, 3-pyridylacetic acid and many secondary and tertiary amines which are capable of forming N-nitroso compounds and amine oxides directly or indirectly.

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8. Brief statement of working hypothesis:

The rate of elimination of nitrogen bases assimilated as a result of tobacco use is dependent upon a variety of factors. These factors include metabolic destruction, excretory routes (renal, respiratory, etc.) and various competitive factors between the parent bases, their metabolites, and structurally related compounds arising from the diet or normal metabolism. It becomes important to determine these relationships since both parent bases and their real or hypothetical metabolites (for example, amine oxides and nitroso compounds) have been implicated in various disease processes. Once the processes and relationships are better understood, one can more easily determine whether or not existing and future health-related claims are sound or unsound. It should also then be easier to treat and study variation between individuals in terms of biological mechanisms and not merely as statistically different phenomena.

9. Details of experimental design and procedures (append extra pages as necessary)

The literature attributes to or implicates nicotine and related pyridine bases a role in production of beneficial effects and detrimental effects. Real or alleged detrimental effects include the enhancement of platelet aggregation that has been attributed to nicotine that is obtained from smoking (Levine, Circ. 48, 619 (1973)) and a possible relationship to carcinogeneses, which includes nitrosation of nornicotine in vivo ("Environment and Cancer"; pp 113-141, 1971) and an abnormal excretion pattern of nicotine metabolites (cotinine and nicotine-N'-oxide) related to bladder-cancer patients (Gorrod, Jenner, Keyzell, and Nikhael, J. Nat. Canc. Inst. 52, 1974). Beneficial effects include consolidation of learning as induced by cotinine and 3-pyridyacetic acid (Essman, 4th Inst. Cong. Pharm.(1969)), and through use of nicotine, nornicotine, and cotinine, a lowering in aggressivity problems (U.S. Pat. No. 3,870,794, Hutchinson et al.). Other possible beneficial effects include an inhibition in lipolysis brought about by 3-pyridylacetic acid and its glycine conjugatate. Comparative studies on biological properties these two nicotine metabolites have recently been described (Bowman et al. IRCS, 3, 65 (1975)). Experimental design and procedures are largely illustrated with the pyridine bases. The general design can however be applied to other nitrogen bases of smoke.

1. Preparation of Compounds: Both isotopic and non-isotopic synthesis can be conducted by procedures based on those previously described ("Tobacco Alkaloids and Related Compounds", U.S. von Euler ed., 1965, p 53 et seq. "Disposition and Fate of Nicotine in Animals" by McKennis). Recent modifications to the original methods have also been described in publications from this laboratory.

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Number 9 continued:

2. Control of Urinary Elimination of Pyridine Bases: Investigations of Isaac and Rand (Eur. J. Pharmac., 8 269 (1969), Langone et al. (Arch. Biochem. Biophys. 164, 536 (1974), Haines et al. (Chem. Pharm. 16 1083 (1974), Falkman et al (Analyst, 100, 99, (1975), and others, including numerous unpublished observations, point to a rapid disappearance of nicotine from the blood of smokers. For many days after smoking, cotinine, allohydroxycotinine and other nicotine metabolites are excreted. The quantity of cotinine appearing in the urine, using the figures of Langone et al. (Biochem. 12, 5025, 1973), appears from all data, as calculated by us, to exceed the amount of cotinine available in the blood.

Since cotinine is a lingering compound and assumed by some to be carcinogenic (Boyland, Planta Med. Suppl. 13 (1968) it is desirable to determine why elimination of cotinine is slow and the nature of the cotinine reservoir in man and animals which is providing a source of urine cotinine beyond that seen from blood levels.

A. Possible Chemical Precursors of Cotinine

Animal data suggest that nicotine alone is not the source of the slowly eliminated and long-lingering cotinine. Blood data and previous radioautographic data (Bowman et al., J. Pharm. Exp. Therap., 143, 301 (1964)) Schmiterlow and Hanson in "Tobacco Alkaloids and Related Compounds", von Euler, ed. p. 75) emphasize the need to look for other sources.

We will, therefore, look for sources of cotinine other than nicotine for cotinine in the urine. Concurrently, one needs to know renal mechanisms for elimination of cotinine and determine whether or not protein binding is an important contributor to the slow release of cotinine. Proper candidates for the extra cotinine include 4-(3-pyridyl)-4-methylaminobutyric acid (McKennis et al., J. Am. Chem. Soc. 79 6342 (1957), cotinine-N-oxide, and nornicotine. To determine the feasibility of such studies, pilot studies have already been conducted in this laboratory with cotinine-N-oxide. Cotinine-N-oxide was determined chromatographically in processed urine and cotinine was isolated as a picric acid salt.

Although preliminary data indicates that cotinine-N-oxide may feed the cotinine reservoir, there is no data on the possible effect of the N-oxide of allohydroxycotinine on the reservoir. The synthesis of the N-oxide of allohydroxycotinine for the contemplated studies will involve the use of the intermediate dibromoticonine (McKennis et al., J. Chem. Soc. 2046 (1973)).

B. Renal Mechanisms

Despite continued focus on the multiplicity of metabolites and continuing need for knowledge on rate determining and competive processes, there has been relatively little said in the literature on the renal mechanisms for elimination of nicotine and its metabolites, aside from the modern investigation of pH effects that were originally put forth by Larson and coworkers many years ago and broadened by the recent studies of Beckett et al. The fact that additional data on renal mechanisms is required becomes very obvious from existing reports of Beckett et al., showing virtually no pH effects on various nicotine metabolites and demonstrable effects only indicated with nicotine (as earlier described by Larson et al.). The methods for the required studies are almost classical in many cases.

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Number 9 continued:

Clearance of some of the quaternary ammonium nicotine metabolites can be compared with the established patterns for the normal dietary constituent trigonelline and N'-methylnicotinamide as described by Beyer et al (Am. J. Physiol. 160 311 (1950)). Both stop-flow free-flow methods can be employed. The amphoteric nature of many nicotine metabolites suggests that both the base transport system and the organic anion transport system will be involved. These notions can be tested by use of Cyamine 863, a potent inhibitor of the base transport system, and with Probenecid, an inhibitor of the organic anion transport system.

Preliminary feasibility studies have been started here on one quaternary ammonium ion metabolite, cotinine methonium ion. The results from this, where clearance of inulin was simultaneously measured, indicate that there is probably an active secretary mechanism for the metabolite and that protein binding is not an important factor. It may be imagined however that protein binding - not yet studied - is a highly significant factor in the slow elimination of cotinine from man and other species. Experimental data on this one point alone as generated in this project should do much to clarify claims that continue to arise.

3. Special Aspects of N-nitrosamine Formation In Vivo: It is apparent that because test results indicate that approximately 85% of the N-nitrosamines thus far tested show carcinogenic activity that all of these substances will be suspect for many years. Leaving aside the reported occurrence of N-nitroso compounds in smoke and tobacco itself, the facilitation of N-nitrosamine formation in vivo can be considered to take place if secondary or tertiary amines (Smith, The Chemistry of Open-Chain Organic Nitrogen Compounds, vol. 1 (1965)) react with nitrite that is obtained from the diet. It is often considered that the stomach is an advantageous spot for such a reaction. For instance, the fact that nicotine is secreted into the stomach makes nicotine a proper candidate, and the hypothetical product would be N-nitrosonornicotine. Easier to see is the reaction to form N-nitroso compounds from the two metanicotine isomers (Sprouse et al., Coresta Abstracts 32, (1972)) and from dihydrometanicotine. These two or three metabolites of nicotine have been studied, in part, in this laboratory, but cis-metanicotine metabolism has been much neglected. The competive nature of biotransformation of these compounds will be studied with the aid of ^{14}C labelled material. Previous informal reports to the Council for Tobacco Research - U.S.A. have indicated that the oxidative series of metabolic reactions for elimination of these three bases is initiated by diamine oxidase and probably inhibited by histamine (competitive) and possibly also inhibited by some the commonly employed antihistamines.

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10 Space and facilities available (when elsewhere than item 2 indicates, state location):

Two laboratories (approximate total of 800 sq. ft.), well-equipped for chemical and pharmacological studies, are available for these studies. In addition, there are two instrument rooms which house spectrographic, chromatographic, and radioactive counting equipment. Animal quarters (shared with others) are available for small mammals and large animals (horses, etc.) are kept in rented areas or at a school animal farm.

List of some major items of permanent equipment available for this work:

Cary recording spectrophotometer, model 11-PM
Grass polygraph, six channel, model 5
Nuclear-Chicago liquid scintillation system, 720 series
Beckman aminoacid analyzer, model 120B
Perkin-Elmer gas chromatograph, model 801
Nuclear-Chicago gas chromatography counting system
Wilken Aerograph Autoprep, model A-700
Preiser Scientific integrator-printer
Wilkens Aerograph 200 (2 each)
Nuclear-Chicago Actigraph III paper radio chromatography system
International preparative ultra centrifuge, model B-35
Vacuum pumps (six of various types)
Warburg Apparatus
Hewlett Packard Model 5700A Gas Chromatograph with integrator
Chemical balances (4 each)
Zeiss photoelectric polarimeter
Cahn electrobalance
Fraction collectors (2 each)
Miscellaneous glass metabolism cages, distillation equipment, chromatography equipment
Radiometer pH meter, O_2CO_2 determinator
Blood oxygenator (local design for organ perfusion)
Varian A-60 NMR apparatus
DuPont Model 830 Liquid Chromatography Apparatus
Varian M-66 Mass Spectrometer
Packard Tri-Carb Tissue Oxidizer
Bechman LS-50 Liquid Scintillation System
Aminco-Bowman Spectrophotofluorometer
Chronolog Platelet Aggregometer with Recorder

Additional medium resolution mass spectrometer service is available with data handling through State Laboratories.

11. Additional facilities required:

If any, these would be determined by the outcome of the investigation.

12. Biographical sketches of investigator(s) and other professional personnel (append).

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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Number 13:

Five Most Recent and Pertinent Publications of Investigators:

1. Bowman, F. J. and Foulkes, E. C., Antidiuretic Hormone and Urea Permeability of Collecting Duets, Am. J. Physiol. 218, 231 (1970).

2. Bowman, F. J., Bowman, E. R., and McKennis, H., Jr., Effects of 3-Pyridylacetic Acid in Rabbit Epididymal Fat Pads, IRCS 3, 65 (1975).

3. McKennis, H., Jr., Bowman, E. R., Quin, L. D., and Denny, R. C., J. Chem. Soc. Perkin Trans. I, 2046 (1973).

4. Bowman, E. R., Chang, R. S. L., Sprouse, C. T. and McKennis, H., Jr., N-3-Pyridylacetylglycine as a Nicotine Metabolite, Abstracts of Papers, 27th Tobacco Chemists' Res. Conf., 1973 p 32.

5. McKennis, H., Jr., Chang, R. S. L., Bowman, E. R. and Wilson, K. L., Jr., Effects of Isomers of Metanicotine on Smooth Muscle, Fed. Proc., 33; 470 (1974).

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Edward R. Bowman, Ph.D.	50%	11,000
Herbert McKennis, Jr., Ph.D.	10%	no request
Faye J. Bowman, Ph.D. (not related to E. R. Bowman)	10%	no request
Technical		
Kendall Wilson M.S.	100%	10,752
Graduate Student (not selected)	ca 50%	3,700
Graduate Student (not selected)	ca 50%	no request
Sub-Total for A		25,452
B. Consumable supplies (by major categories)		
Animals and animal care		3,500
Chemicals and glassware		1,900
Isotopes		1,750
High Speed liquid chromatography columns		450
Sub-Total for B		7,600
C. Other expenses (itemize)		
Communication		250
Travel (Scientific paper presentation and Conferences on project related material)		625
Mass spectrometer service		1,200
Sub-Total for C		2,075
Running Total of A + B + C		35,127
D. Permanent equipment (itemize)		
N/A		
Sub-Total for D		---
E. Indirect costs (15% of A+B+C)		5,269
Total request		40,396

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	31,000	8,500	3,000	3,000	6,375	51,875
Year 3	34,000	8,500	3,000	---	6,825	49,325

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Gifts	Annonymous and American Tobacco Co.	$33,000	June 30, 1974 July 1, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Cardiovascular Effect of Nicotine Metabolites	N.I.H.	101,879	May 1, 1975 June 30, 1978

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert McKennis, Jr.

Signature Herbert McKennis, Jr. Date 15 Apr 1975

Telephone 804 (Area Code) 770-4406 (Number) N/A (Extension)

Checks payable to

Walter Lossing, Comptroller

Mailing address for checks

Medical College of Virginia

1200 East Broad Street

Richmond, Virginia 23298

Responsible officer of institution

Typed Name Martha Bell Conway, Administrator

Title Research Grants and Contracts

Signature Martha Bell Conway Date 4/24/75

Telephone (804) (Area Code) 770-4443 (Number) (Extension)

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R: REDACTED MATERIAL

Dr. Herbert McKennis, Jr.
Medical College of Virginia
Richmond, Virginia

Born: REDACTED
Citizen

Education:

Harvard S. B. 1938 (field of concentration - chemistry).

Polytechnic Institute of Brooklyn 1939 - 1942 - graduate student in chemistry.

Cornell University Ph.D. 1945 (major subject - biochemistry; minor subjects - physiology and pharmacology).

Experience:

1955 - present	Professor of Pharmacology Medical College of Virginia
1960 -	Visiting Professor Institute of Physiology, University of Chile
1953 - 1955	Associate Professor of Research Pharmacology Medical College of Virginia
1949 - 1953	Head, Basic Sciences-Research Department Naval C. E. Research and Evaluation Laboratory Port Hueneme, California (and Solomons, Maryland)
1948 - 1949	Associate Professor of Biochemistry Department of Surgery Medical College of Virginia
1946 - 1948	Instructor in Physiological Chemistry School of Medicine, The Johns Hopkins University
1945 - 1946	Assistant Professor of Chemistry Medical College of Virginia
1942 - 1945	Assistant in Biochemistry Cornell University Medical College
1940 - 1942	Chemist Ciba Pharmaceutical Products, Summit, New Jersey
1938 - 1939	Chemist Nuodex Products Company, Elizabeth, New Jersey

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R: REDACTED MATERIAL

Honorary Member:

REDACTED

Professional Societies:

REDACTED

REDACTED

REDACTED

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R: REDACTED MATERIAL

Edward R. Bowman
Medical College of Virginia
Richmond, Virginia

Born: REDACTED
Citizen

Education:

Concord College B.S. 1952 (Biology - Chemistry).

West Virginia University 1953 (Physiology).

Duke University 1955-56 (Graduate Student in Physiology).

Medical College of Virginia Ph.D. 1963 (Pharmacology).

Experience:

1961 - present	Research Associate Department of Pharmacology Medical College of Virginia Richmond, Virginia
1958 - 1961	Graduate Student, Major - Pharmacology Minor - Physiology & Biochemistry Medical College of Virginia Richmond, Virginia
1956 - 1958	Research Assistant Department of Pharmacology Medical College of Virginia Richmond, Virginia
1955 - 1956	Graduate Student, Major - Physiology Minor - Anatomy Duke University Durham, North Carolina
1954 - 1955	Bacteriologist State Department of Health Richmond, Virginia
1952 - 1953	Graduate Student, Physiology West Virginia University Morgantown, West Virginia
1952	Student, Biology & Chemistry Concord College Athens, West Virginia
1950 - 1951	U.S. Army
1947 - 1950	Student, Biology & Chemistry Concord College Athens, West Virginia
1944 - 1946	U.S. Army

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Professional Societies:

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Curriculum Vitae

1. Personal Information:

1.1 Alta Faye Bowman (Maiden Name: Johnson)
1.2
1.3
1.4 REDACTED
1.5
1.6 REDACTED

1.7 Medical College of Virginia
Department of Pharmacology
Room 434, McGuire Hall Annex
Richmond, Virginia 23298
(703) 770-4683

2. Licensure:

2.1 N/A
2.2 N/A

3. Education:

Vanderbilt University
Nashville, Tennessee Ph.D. 1967

Tennessee Polytechnic Institute
Cookeville, Tennessee M.A: 1962
B.S. 1961

4. Military Service Record:

None

5. Postdoctoral Training, or Special Experience:

None

6. Academic Appointments or Other Significant Work Experience:

Research Associate
Department of Pharmacology
Medical College of Virginia
Richmond, Virginia 1970-Present

Research Assistant
Kettering Laboratory
University of Cincinnati
Cincinnati, Ohio 1967-1969

7. Membership - Scientific, Honorary and Professional Societies:

REDACTED

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CURRICULUM VITAE

NAME: Kendall Louis Wilson, Jr.

PERMANENT ADDRESS:

DATE OF BIRTH: REDACTED

PLACE OF BIRTH:

MARITAL STATUS: REDACTED

SECONDARY EDUCATION: Marion High School
Marion, Maryland

HIGHER EDUCATION:

Dates	Institution	Degree
1966-1970	Randolph-Macon College Ashland, Virginia	B.S. in Biology
1970-1974	School of Graduate Studies Medical College of Virginia Richmond, Virginia	M.S. in Pathology

Thesis Title: "An Investigation of Momordica balsamina as an Antifertility Agent"

POSITIONS HELD:

1967-1968	Laboratory Assistant - General Biology Randolph-Macon College, Ashland Virginia
1969	Laboratory Instructor - Histology Randolph-Macon College, Ashland, Virginia
1970-1973	Laboratory Specialist, Clinical Pathology (Hematology and Clinical Microscopy Labs) Medical College of Virginia Richmond, Virginia
1973-Present	Research Assistant - Department of Pharmacology Division of Biochemical Pharmacology Medical College of Virginia Richmond, Virginia

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1973-Present Laboratory Specialist, Night Service Clinic
Medical College of Virginia
Richmond, Virginia (10 hrs/week)

HONORS:

MEMBERSHIP: REDACTED

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#1035 - NEURATH

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 19, 1975

Grant application No. 1035

PHARMACOLOGY

To: The committee comprising Drs. Bing, Huebner, Jacobson and Wyatt

Subject: Georg B. Neurath, Ph.D., Hamburg, Germany
New application No. 1035
"Interaction among Smoking Habit and Drub Bioavailability

History:

In October 1972 a three-year plan was approved by the SAB (Grant No. 981) for studies entitled "Kinetics of Nitrosamine Formation in Tobacco Smoke". The second renewal (Grant No. 981R2), at a level of $46,120, terminates December 31, 1975.

The present application was handled as Case No. 314, which tentatively requested a total of $172,000 (3 year period) for a study involving a broad spectrum of drugs. At the suggestion of CTR that a pilot study at a modest budgetary level might be considered, the present application limits the number of drugs to be studied.

Request

Application No. 1035 requests $53,130 for a one year pilot study.

Document attached

Application dated June 6, 1975 (9 pages).

D.S.

DS:wg
Encl.

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#1035

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A. INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUN 19 1975

Application for Research Grant

(Use extra pages as needed)

Date: June 6, 1975

1. Principal Investigator (give title and degrees):

Dr. Georg B. Neurath

2. Institution & address:

INSTITUT FÜR BIOPHARMAZEUTISCHE MIKROANALYTIK

2000 H a m b u r g, 56, Hexentwiete 32, F.R. Germany

3. Department(s) where research will be done or collaboration provided:

1003540824

4. Short title of study:

Interaction among Smoking Habit and Drug Bioavailability

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 1 year

7. Brief description of specific research aims:

Investigation on the influence of habitual cigarette smoking on the bioavailability and metabolic changes of a choice of important and widespread drugs on the basis of a feasibility study. The project is proposed to comprise the determination of blood levels, biological half lives and urinary excretion rates of aminopyrine caffeine, and chloramphenicol comparing a group of ten, seven male and three female, heavy smokers (30 and more cigarettes per day) and a group of ten non-smokers with the same sex distribution respectively.

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8. Brief statement of working hypothesis:

In 1972 A.H. Conney and J.J. Burns (Science 178 (1972) 576) gave e review on known interactions among environmental chemicals and drugs, based on stimulatory and inhibitory effects of such factors on the microsomal liver enzyme systems essential for drug metabolism, and as a consequence for the bioavailability of drugs, i.e. for the effective dose of them in the organism.
Among those environmental factors known to influence the bioavailability of drugs tobacco smoke and nicotine are mentioned, for instance to increase the metabolism of phenacetine in smokers compared to non-smokers (E. Pantuck, R. Kuntzmann, and A.H. Conney, Science 175 (1972) 1248). Exposition to cigarette smoke is also known to increase the benzopyrene hydroxylase activity in the lung, the liver, the intestine, and the placenta of rats (A. Loh, A.H. Conney, Life Sci. 10 (1971) 215. The activity of this enzyme as well as aminoazo dye N-demethylse were greatly increased in term placentas of individuals who smoke cigarettes (R.M. Welch et al., Science 160 (1968) 541).

It therefore should be clarified, whether there is an influence of heavy smoking on the effective dose of important and widespread drugs.

9. Details of experimental design and procedures (append extra pages as necessary)

Because of the high standard of analytical procedures, the bioavailability of drugs, the effective dose in the organism,can be directly measured in many cases. Gas chromatography and high pressure liquid chromatography are means which enable to determine minute amounts of drugs in body fluids and human excretions.

Heavy smokers and non-smokers shall be compared as to their respective potential as to uptake, metabolism, and excretion of normal doses of drugs.

Comparison of the biological half life of drugs and metabolites in smokers and non-smokers shall be performed by statistical evaluation of the resulting data.

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DR. GEORG B. NEURATH
Vertragsforschung und Beratung
INSTITUT FÜR BIOPHARMAZEUTISCHE MIKROANALYTIK

THE COUNCIL FOR TOBACCO
RESEARCH - U.S.A., INC.
110 East 59th Street
New York, N.Y. 10022

2000 Hamburg 56
Hexentwiete 32
Telefon (040) 81 60 40

June 6, 1975

Interaction Among Smoking Habit and Drug Bioavailability

In 1972 A. H. Conney and J. J. Burns (Science 178 (1972) 576) presented a review on known interactions among environmental chemicals and drugs, based on stimulatory and inhibitory effects of such factors on the microsomal liver enzyme systems essential for drug metabolism, and, as a consequence, for the bioavailability of drugs, i.e. for the effective dose of them in the organism.

Among those environmental factors known to influence the bioavailability of drugs and other xenobiotics tobacco smoke and nicotine are mentioned.

Thus, the metabolism of phenacetin, an important analgesic and antipyretic, was found to be increased in smokers compared to non-smokers (E. Pantuck, R. Kuntzmann, and A.H. Conney, Science 175 (1972) 1248).

Exposition to cigarette smoke is also known to increase the benzopyrene hydroxylase activity in the lung, the liver, the intestine, and the placenta of rats (A. Loh, A.H. Conney, Life Sci. 10 (1971) 215). The activity of this enzyme as well as aminoazo dye N-demethylase were

Postscheckkonto: Hamburg 517 93-200 (BLZ 200 100 20) · Dresdner Bank 6 061 059 (BLZ 200 800 00)
Vereinsbank in Hamburg 15/03 457 (BLZ 200 300 00)

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greatly increased in term placentas of individuals who smoke cigarettes (R. M. Welch et al., Science 160 (1968) 541).

Nicotine tolerance occurring in smokers may be explained by the enhancement of nicotine metabolism by enzyme induction in smokers (A. H. Beckett and E. J. Triggs, Nature 216 (1967) 587), and even long term marijuana smokers metabolize tetrahydro cannabinol more rapidly than non-smokers (L. Lemberger, N. R. Tamarkin, J. Axelrod, and J. J. Kopin, Science 173 (1971) 72).

I therefore should like to propose on the basis of a feasibility study to investigate blood levels, biologic half-lives, and urinary excretion rates of

aminopyrine (Pyramidon)	antipyretic, analgesic
caffeine	CNS, respiratory, cardiac stimulant
chloramphenicol (Paraxin)	antimicrobial.

Based on the experience gathered in a number of studies on the bioavailability of drugs in human beings, which were performed for the pharmaceutical industry, our institute is expert to investigate the influence of habitual cigarette smoking on the bioavailability and metabolic changes of this choice of important and widespread drugs.

The anticipated duration of this feasibility study is one year. The total estimated costs required, including development or adaptation of the analytical procedures, volunteers as test persons and medical care, determinations in blood and urine, pharmacokinetic and statistical evaluations, and overhead are $53,130.

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Plan of Work

1. Analytical Procedures

The analytical procedures for drugs and major metabolites will be developed or be adapted to the expected levels of drugs, metabolites, and conjugates in blood and urine.

Gas chromatography or high pressure liquid chromatography will be applied to according to the feasibility in the respective cases.

2. Test Persons

Healthy men and women, smokers and non-smokers respectively, will be selected from volunteers experienced in drug metabolism studies. Groups of heavy smokers (more than 30 cigarettes per day) and non-smokers, each of seven male and three female volunteers, will be recruited.

3. Administration of Drugs

In repeated single dose experiments the three selected drugs will be administered according to the indications of the respective producers and under medical observation and care like in clinical tests.

4. Blood Levels

Blood samples will be taken from the test persons at 0, 30, 60, 120, 240, and 480 minutes after administration (or time intervals which turn out to be suitable for the drug under consideration).

According to the distribution of the drug, the levels will be determined in whole blood, serum, or plasma respectively.

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5. Urinary Excretion

Urine samples will be collected of the following time intervals after administration: 0, 0 - 4, 4 - 8, 8 - 12, and 12 - 24 hours.

The total excretion rates of drugs and metabolites will be determined comprising the total excreted amounts including conjugates (after treatment with ß-glucuronidase).

6. Biologic half-lives

The biologic half-lives of the drugs will be determined on a personal basis and be compared among smokers and non-smokers. They will be also evaluated separately for men and women.

7. Urinary Excretion Rates

The total excretion rates of the drugs will be compared among smokers and non-smokers being determined on a personal basis and evaluated separately for men and women.

8. Statistical Evaluation

Blood levels, biologic half-lives, and urinary excretion rates of smokers and non-smokers, if necessary separately for men and women, will be compared by means of a statistical evaluation in cases of the three proposed drugs.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Gas chromatography and liquid chromatography equipment of all kinds.
Equipment for tracer studies available.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

see former application and project

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see former reports on formation of nitrosamines

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Chemist	30	$ 8,500
Chemical Engineer	100	$17,500
Technical		
Technician	25	$ 2,300
Sub-Total for A		$28,300
B. Consumable supplies (by major categories)		
Chemicals		$ 1,800
GC- and CC-supply, glass		$ 2,800
Small apparates		$ 3,500
Supply gases for GC		$ 2,300
Sub-Total for B		$10,400
C. Other expenses (itemize)		
Test persons and medical care		$ 5,800
Statistical calculations		$ 1,000
Travel expenses		$ 700
Sub-Total for C		$ 7,500
Running Total of A + B + C		$46,200
D. Permanent equipment (itemize)		
None		
Sub-Total for D		0
E. Indirect costs (15% of A+B+C)		$ 6,930
Total request		$53,130

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/mkpl0000

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
KINETICS OF THE NITROSAMINE FORMATION IN TOBACCO SMOKE	CTR No. 891R2	$46,120	expires Dec.31,1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Georg B. Neurath

Signature Georg B. Neurath Date June 6,1975

Telephone F.R.G. (040) 81 60 40
Area Code Number Extension

Checks payable to

Dr. Georg B. Neurath

Mailing address for checks Hexentwiete 32

2000 H a m b u r g 56, F.R.Germany

Responsible officer of institution

Typed Name _____

Title _____

Signature _____ Date _____

Telephone _____
Area Code Number Extension

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